THE UNIVERSITY OF ALBERTA

THE FUNCTIONAL MORPHOLOGY OF THE REPRODUCTIVE SYSTEMS AND THE LARVAL DEVELOPMENT OF TWO INTERTIDAL SNAILS, LITTORINA SITKANA (PHILIPPI, 1845) AND LITTORINA SCUTULATA (GOULD, 1849) WITH SPECIAL REFERENCE TO THE SPERMATOZOA

by

© JOHN BUCKLAND-NICKS

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ABBREVIATIONS

The following important abbreviations have been used in the thesis besides those specified in the text:

um = microns

mu = millimicrons

S-V = Seminal Vesicle(s)

75A = 75 Angstroms

55A = 55 Angstroms

revs./sec. = revolutions per second

ABSTRACT

Some aspects of the reproductive biology of two sympatric species of Littorina { L.sitkana and L.scutulata } have been investigated, including: the functional morphology of the male and female reproductive systems; the breeding seasons; and oviposition and larval development. Emphasis has been placed on the functional morphology of the spermatozoa, which has involved considerable use of the transmission and scanning electron microscopes. These studies include: spermatogenesis and fine structure; motility; transfer; storage; resorption. Some of the most significant findings are listed below.

1. The Functional Morphology of the Male System

A. The penis of L.sitkana bears along its ventral edge 14 mammiliform glands , which produce both apocrine and eccrine secretions. In L.scutulata the glands are absent but instead there is an elongate dorsal process, which is prehensile.

B. The spermatozoa of the two species are virtually identical. During spermiogenesis a 'ring centriole' (annulus) forms from the distal centriole and migrates along the flagellum to the mitochondrial-tail junction. An infolding of the plasma membrane at this junction allows the tail to beat perpendicularly to the main

flagellar axis. Observations on the form of beat of stationary and swimming sperm have provided the basis for an hypothesis to explain the unusual propulsive mechanism. The sperm can attain speeds of 185 um/sec. in media of widely ranging viscosity.

C. The centriolar complex (basal body + distal centriole) is formed by an alignment process, in which the procentriole (proximal) rotates from a position perpendicular to the distal centriole, to one coaxial with it. Subsequently the procentriole fuses with the distal centriole and forms the basal body.

D. Pseudopodia of nurse cells form desmosome-like junctions with developing sperm. The nurse cells detach from the testicular wall, the nuclei degenerate and secretion droplets form in the cytoplasm. Later the clump of attached spermatids becomes grouped unilaterally. Mature nurse cells with sperm (spermatozeugmata) are stored in the seminal vesicles.

F. Phagocytosis of sperm in the seminal vesicles occurs both directly by epithelial cells or indirectly by macroapocrine secretions of the cells which are later resorbed. Heterophagic vacuoles are digested by the lysosome system of the cell.

2. <u>Functional Morphology of the Female System</u>

A. The bursa copulatrix receives the sperm packet

and serves as a temporary sperm storage organ. Its secretions destroy the seminal fluids and nurse cells, and liberate the sperm. Old sperm are digested extracellularly.

B. Long term storage of sperm occurs in the receptaculum seminis. The sperm heads become embedded in invaginations of the epithelial cells of this organ, with tails beating synchronously in the lumen.

C. A modified technique for micro-injection of snail reproductive systems, developed in this study, has enabled a more complete understanding of the complex pathway of the pallial oviduct and how the glands coordinate to secrete the various egg envelopes.

D. The pallial oviduct of L.sitkana consists of: receptaculum seminis; albumen gland (covering gland); capsule gland; jelly gland and ovipositor. In L.scutulata the system is reduced to covering gland and capsule gland. The formation of the egg envelopes in the two species is described.

E. The glands of the pallial oviduct are merocrine and extrude their secretions in small depressions between ciliated epithelial cells, whose apices cover most of the luminal surface. The granular endoplasmic reticulum plays an important role in the synthesis of the glandular secretions.

3. <u>Oviposition and Larval Development</u>

A. *L.scutulata* is a planktotrophic developer and spawns in the summer, whereas *L.sitkana* is a benthic (lecithotrophic) developer and spawns in two seasons, spring and autumn. Breeding is considered to be under the influence of a number of environmental factors, especially photoperiodicity.

B. The larval development of each species is described with special emphasis on the hatching mechanisms. *L.scutulata* veligers hatch by mechanical and possibly osmotic factors. *L.sitkana* juveniles utilise the foot, larval shell and in later stages, the radula, to rupture the egg covering and capsule wall. Rows of tubercles on the larval shell form an abrasive surface, which may facilitate hatching.

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INTRODUCTION

The philosophy of the ancient Egyptians, as set down by Hermes Trismegistus in the 'Kybalion', describes seven Principles that govern the universe. One of these is the Principle of Gender which applies to the creative, or reproductive, processes in the universe. Stated simply, it is that the masculine (positive) aspect is constantly attracted to the feminine (negative) aspect. The Principle is said to apply, whether one is concerned with universes, organisms, gametes, or atoms. In the animal world, one can visualise natural selection as having acted within such an empirical force by favouring adaptations to specific environmental niches, thereby producing great diversity in the reproductive strategies of different organisms. None the less, where the environment has remained relatively constant, such as the cellular and subcellular environment of reproductive cells, one can expect that basic similarities in functional morphology would be retained throughout the Metazoa. Studies of cellular processes in one phylum might lead to an understanding of similar processes in another, quite different, phylum.

It is not the purpose of this thesis to try and prove or disprove this philosophy but merely to bring to mind some far-reaching possibilities such as this one and leave them open for contemplation. However, an attempt has been made to draw useful parallels between the functional morphology of

certain specific cells in Littorina with comparable cells in other animal phyla. Specific examples are, the similarity between sperm resorption in Littorina and any other intracellular digestive process involving lysosomes; or the similarity between littorinid sperm structure and that of many internally fertilising organisms both within the Mollusca and in other phyla.

Another purpose of this work concerns the basic problem "How does the sperm reach the egg?". In many aquatic organisms, sperm are shed freely into the water and they must swim actively to reach the eggs and fertilise them. The sperm of all these organisms have a prominent head and long filamentous tail and are considered 'primitive'. However many other aquatic species copulate and have internal fertilisation. In these organisms the sperm are usually bound up in sperm packets (spermatophores) before delivery to the female. Sperm that fertilise the eggs inside the genital tract usually swim efficiently in the more viscous fluids of the oviduct, whereas primitive sperm do not. Species that have internal fertilisation produce streamlined, filamentous sperm that often have structural modifications such as an enlarged, elongated middle piece and accessory fibres, to meet the greater energy requirements for movement through the viscous oviductal fluid. In molluscs both modes of fertilisation occur. Littorina belongs to the group that has internal fertilisation, which includes the more advanced Gastropoda

and Cephalopoda. My studies on spermatogenesis, sperm motility, and the detailed morphology of the genital tracts of both sexes have contributed to the understanding of sperm transportation and storage, site of fertilisation, deposition of albumen, formation of egg envelopes, and the removal of excess sperm.

In connection with the study of gametes and the reproductive tracts, oviposition and larval development have also been examined. It is a well-known fact that the size of the ova has a profound effect on the type of larval development and that this in turn dictates the breeding cycle. Thus, the larval development and breeding cycle are natural extensions to the study of gametes. Moreover, it provides a useful background for examining the question of evolutionary adaptations of different developmental patterns in closely-related, sympatric species such as L.sitkana and L.scutulata.

Specific objectives and relevant literature are addressed in the individual sections, either in the introduction, or discussion, or both.

The third purpose of this thesis, is that which many researchers hope for their work, to create new stimulus and discussion among scientists, especially those studying molluscan biology.

THE FUNCTIONAL MORPHOLOGY OF THE REPRODUCTIVE SYSTEMS

MATERIALS AND METHODS

All the animals used in this study were collected from the intertidal tide pools at False Bay, San Juan island, Washington (PLATE 1). The snails are easily recognised in the field, since _L.sitkana_ has a flattened, roundish shell with grooves following the whorls and a large opercular aperture (PLATE 2A,B), whereas _L.scutulata_ has a more conical, pointed shell with a smooth surface and a smaller opercular aperture (PLATE 2C,D). Each species has a variety of colour morphs with some overlap. Thus, in spite of the fact that the name 'scutulata' means 'speckled' or 'mottled', shell colour is not a good parameter for distinguishing them. Caged populations, numbering about two hundred, were maintained for two and a half years. Animals were taken back to the laboratory and immediately fixed for light and electron microscopy. These methods follow:

1. Light Microscopy

a. Fixation

Fresh material was fixed in Bouin's, Baker's calcium-formaldehyde or Smith's formol-bichromate. Of these Bouin's

proved to be the most useful for routine histological work. Fixed material was dehydrated in an ethanol series. Picric acid was removed by adding a few drops of 3% ammonium hydroxide to several changes of 70% ethanol (this reduced brittleness in the block). It was essential to use a double embedding technique as the material was found to be too brittle without it. The technique outlined by Humason (1967) proved to be satisfactory. In this, a series of changes of a 1% solution of celloidin in methyl benzoate follows absolute ethanol for a total time of up to 72 hours. The tissue subsequently, is taken through three changes of pure benzene, one change of 1:1 benzene: paraplast wax and two changes of pure paraplast, before finally embedding in pure paraplast wax (M.P. 56°C). Sections were cut at 7-10um .

b. Staining

A variety of general stains were tried, but of these the most successful were Delafield's haematoxylin and eosin, and Masson's trichrome. The latter produced beautiful colours in the sections and was good to work with. The Alcian Yellow - Alcian Blue technique was used to differentiate between acid mucopolysaccharides (Ravetto, 1964; Peute and Van de kamer, 1967; de Jong Brink, 1969). It was hoped that this would enable easier identification of different glands.

Sections were mounted in Canada Balsam and observed with either a Vickers, a Wild, or a Zeiss compound

microscope.

2. Electron Microscopy

Material was fixed in the following ways:

a. Primary fixation with 2.5% glutaraldehyde (Fisher Scientific Co.) in 0.4M Millonig's phosphate buffer (pH 7.4) for 1.5 hours at 20°C. Secondary fixation with 1% osmium tetroxide in 2.5% sodium bicarbonate buffer (pH 8.0) for 1 hour at 0 - 4°C (Pease 1964).

b. Primary fixation with 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) plus impurified ruthenium red for 6 hours at 20°C (J. Luft, 1961 - modified by M. Cavey, 1971). Secondary fixation with 2% osmium tetroxide buffered with 2.5% bicarbonate (pH 7.4), for 1 - 2 hours at 0 - 4°C.

c. Primary fixation was 2.5% glutaraldehyde (Fisher Scientific Co.) in filtered sea water (pH 8.0) for two hours at 15°C, followed by a sea water rinse and secondary fixation with 2% osmium tetroxide in filtered sea water for one hour at 15°C. The tissue was dehydrated in an ethanol series at 20°C, transferred to two 15-minute changes of propylene oxide and flat embedded in Durcupan ACM (Fluka, Switzerland). Sections with a silver-grey interference colour were cut on a Porter Blum MT-2 ultramicrotome and picked up on 150 mesh formvar-coated grids. Sections were stained with 2% aqueous uranyl acetate for two hours at 20°C, and observed with a Phillips EM200. For light

microscopy araldite sections were cut at 0.5um and stained with Richardson's stain (Richardson et al., 1960).

Fixatives a, b, and c were used in Spring and Summer 1972 and 1973, for fixation of eggs and embryos and parts of the reproductive systems of both snails. Fixatives a and c were found to be superior and were used in subsequent fixations.

3. <u>Scanning Electron Microscopy</u>

Tissues were fixed in 2.5% glutaraldehyde in 0.4M Millonig's phosphate buffer for 12 hours at 20°C, followed by post-fixation in 1% osmium tetroxide buffered with 0.4M phosphate buffer for 1 hour at 20°C. The tissues were dehydrated in an ethanol series. Absolute alcohol was gradually replaced by amyl acetate in a step-wise process over a period of 90 minutes, until the tissue could be transferred to pure amyl acetate. The material was taken to the S.E.M. Laboratory, dried by the critical point method (Anderson, 1951) and mounted on aluminium studs. The surface of each stud was rotary shadowed, first with carbon, and then gold, to a thickness of 75-125A and examined in a Cambridge S4 Stereoscan, scanning electron microscope.

Non-perishable materials, such as larval shells, were placed in a solution of bleach until superfluous tissues had been dissolved. They were then washed, air-dried, mounted on aluminium studs and processed in the same way as before.

4. Sperm Motility

Mature sperm were obtained from ripe seminal vesicles in sea water. Samples were pipetted onto a depression slide beneath a coverslip. Observations were made on dark field phase with a Zeiss photomicroscope fitted with a high frequency stroboscope (courtesy Dr.R. Strathmann) at 500x magnification and filmed with a Bolex H16M motion picture camera, 24 frames/sec. The viscosity of the sperm medium was increased by adding minute quantities of sperm, either to drops of oviductal fluid, or to a solution of Protoslo (Carolina Biological Supply Co.) in sea water; solutions ranged from 5-30% Protoslo. 12% Protoslo solution has similar viscosity to oviductal fluid. The velocity of sperm was calculated both directly using a stopwatch and indirectly from cinematographic recordings.

5. Vinyl Acetate Injection Technique

It is difficult to reconstruct a three-dimensional model of a system of ducts from a series of two-dimensional sections without making gross errors and yet most reconstructions of invertebrate systems have been made in this way. Plastic injection has been employed for many years in medical laboratories for replicating vascular systems in various organs of the body (Smith, 1963; Tompsett, 1969; Bradbury, 1971). In 1967 Fankboner injected vinyl acetate

into molluscan alimentary systems and obtained detailed replicas of the finely branching tubules. He injected plastic directly into the animal's mouth by using a hypodermic syringe and needle. It is not possible to use this technique where one encounters minute orifices and delicate tissues such as are found in the molluscan reproductive system. This section describes a technique which has been successfully used to replicate the reproductive system of Littorina. It is hoped that this technique can be used in many other small invertebrates to trace and hence reconstruct organ systems of tubular, or vascular, nature.

Micropipettes were drawn out by hand to 0.25 mm, at each end of 5 cm lengths of 2 mm glass tubing, using a tiny bunsen flame. 40 cm of P.E. 60 intramedic polyethylene tubing (Clay Adams, U.S.A.) was pushed over one end of a micropipette and secured with sealing wax. The pipette was led through a 15 cm piece of 0.4 mm diameter glass tubing and the latter secured in the clamp of a micro-manipulator (PLATE 3A), such that the micropipette could slide freely inside the glass tubing. To the other end of the polyethylene tubing was attached a No.22 gauge hypodermic needle and a 5 ml disposable syringe containing 2ml of red vinyl acetate (Ward's Natural Science Estab. Inc.).

A female snail was selected and the upper 3 whorls of the shell carefully removed by cutting through with blunt

scissors. The columella muscle was severed and the snail pulled out by gripping the operculum with forceps. The animal was immediately decapitated and pinned to a dissecting dish by its foot and operculum. A second pin was pushed between the whorls of the digestive gland complex. The anterior end of the reproductive tract was severed from the body wall so that the loop of a human hair could be pushed over its tip beyond the gonopore. The micropipette was lowered into position in front of the gonopore, using the micro-manipulator controls. The micro-adjuster on the injection apparatus was carefully rotated to force vinyl acetate to the end of the micropipette, so that almost all the air had been expelled from the apparatus. The pipette was then inserted in the gonopore and secured by tying off the hair (PLATE 3A). Vinyl acetate was run in smoothly and continuously until a slight backing pressure could be detected when, immediately, the flow of plastic was cut off. The process was best observed by mounting a dissecting microscope over the micropipette and watching the rate of flow of plastic into the animal.

If the plastic was injected too slowly it set prematurely and the reproductive system remained only partially filled. This could be seen externally since the outer whorls of the oviduct, close to the surface, remained colourless and therefore unfilled. If the plastic was injected too rapidly the tissue burst and the system leaked. The time required to fill the genital tract of a snail 1 cm

long is 2 - 5 minutes.

The replica solidified in 2 hours. After gently removing the pins holding the animal, the micropipette was cut free from the polyethylene tubing and the whole animal immersed in 10N potassium hydroxide, for about 2 days. This dissolved all traces of tissue and left the plastic replica attached to the glass pipette. The replica was carefully washed and dried before taking close up photographs (PLATE 3B, C) Replicas may be stored in vials containing distilled water.

THE MALE REPRODUCTIVE SYSTEM

PART 1: GENERAL MORPHOLOGY

Observations

The testis is composed of a mass of pale orange, testicular tubules, which interdigitate with lobes of the digestive gland. The tubules drain into a common vas deferens, which becomes expanded and coiled to form the seminal vesicles (vesiculae seminales), just below the pericardium. There is no gonopericardial duct in the male of either species. During the breeding season, the seminal vesicles are packed with sperm and have a pearly white appearance. If some of this white fluid is extracted, numerous sperm are seen protruding from large, round, nurse cells containing spherical droplets.

Distally, the vas deferens becomes a narrow muscular tube, which acts as a sphincter regulating the flow of sperm to the prostate. The prostate is a pinkish gland which runs the length of the mantle cavity, beneath the rectum. It is closed, forming a tube for a short distance at the proximal end, beyond which it is open to the mantle cavity. The gland is divided into left and right lobes. The walls of both lobes are folded, providing a greater surface area for secretion. Folding is much more extensive in the right lobe, which is also thicker. A lateral fold of tissue from each

lobe projects across the lumen and together they form a ventral channel.

The prostate is composed of three types of cells, two of which are glandular and the third is a ciliated epithelial supporting cell. One gland cell type produces a large secretion which is probably a mucoid substance staining blue with Masson's trichrome (PLATE 6A). The other produces a finer secretion which is probably a proteinaceous substance staining red with this technique (PLATE 6B).

A mass of spermatozeugmata are mixed with the prostate secretions into a mucus-bound sperm packet, which collects in the ventral channel and is passed along it, by ciliary action. This sperm packet, although only loosely held together by mucus, will be referred to as a spermatophore in this study. At the distal end of the prostate a small flap of tissue projects from the right lobe across the lumen, forming a continuation of the ventral channel. From here an open ciliated groove (seminal groove) passes beside the head, and along the dorsal surface of the penis.

The penis is a muscular organ, formed from the foot, and containing numerous blood sinuses internally. During copulation it is expanded by haemolymph engorgement so that it projects upwards from the right hand side of the head. The morphology of the penis differs markedly in the two species and so they will be described separately.

L.sitkana

The penis is about 3 mm long, when not in use, with a small bifurcation at its tip. A row of mammiliform glands are arranged along the ventral edge (PLATE 7A). These glands are brought into close contact with the internal surface of the female mantle cavity and gonopore region during copulation. Similar glands have been observed in some other species of Littorina.

Each penial gland (papilla) is composed of a number of multicellular glands which produce heterogeneously dense granules (PLATE 6C, D). The secretions produced by each gland pass into a fine elongate process, which penetrates a thick muscle layer before emptying its contents into the lumen of the papilla (PLATES 6D; 34D). Each multicellular gland is surrounded by a thin layer of muscle, which is approximately one cell thick.

Contraction of the muscle sheaths, together with the thick muscle layer surrounding the lumen, forces the secretions out of the papillae onto the ventral surface of the penis, where they are spread out by ciliary action. The muscles of the papillae can precisely control its orientation with the wall of the female mantle cavity. Besides these gland cells there appears to be another type of secretory cell which is of the apocrine type. This cell

has a dense border of microvilli, some of which appear to fill with secretions, forming bulb-shaped extensions that pinch off at the base, freeing them into the lumen (PLATE 6C). This process also occurs in several types of mammalian secretory cell and was reviewed by Kurosumi (1961). A third cell type is the ciliated epithelial supporting cell, which presumably assists in mixing secretions and moving them out of the papilla lumen. Details of the fine structure of the penial glands of Littorina have not been worked out. A scanning electron micrograph of the penis and penial glands illustrating the external morphology, is shown in PLATE 7A .

L.scutulata

The penis of L.scutulata is quite different because there are no mammiliform glands. Instead the tip bifurcates into two processes, the dorsal one of which is much longer than the ventral, and also is prehensile. The seminal groove runs along the dorsal surface to the tip of this elongate process. A scanning electron micrograph of the penis is illustrated in PLATE 7B.

During copulation, in either species, the male crawls over the right hand side of the shell of the female, such that their two right edges are together. The penis is inserted into the gonopore and the dorsal process (furca) empties the spermatophore into the bursa copulatrix (PLATE 7D). If the snails are undisturbed, copulation times vary

from fifteen minutes to several hours.

Two males (L.sitkana) have been found with spermatophores contained in a pocket, at the tip of the penis, formed by the dorsal closure of the seminal groove. When some of the spermatophore was removed it was found to contain a large number of immature and some mature sperm. The nurse cells appeared to have separated and were in the process of degeneration. It is not known how long storage occurred, nor what function it might serve. To my knowledge this phenomenon has not been reported previously.

Discussion

The marked differences in penis morphology between the two snails is probably related to its function as a holdfast during spermatophore deposition. It has been suggested previously that the secretions of the penial glands serve an adhesive function (Linke, 1933: Fretter and Graham, 1962). The following observations support this idea:

1. Male L.sitkana take longer to remove the penis when disturbed after prolonged copulation, than do L.scutulata.

2. The mammiliform penial glands (papillae) can execute precise orientation movements with an adjacent surface such as the mantle wall of the female.

3. The dorsal furca of the penis of L.scutulata is

prehensile and will hold onto a dissecting needle that touches it. This furca can be inserted much deeper into the female bursa than can the equivalent in *L.sitkana*.

It is suggested that the elongate furca with its prehensile properties replaces the need for penial glands with their adhesive functions. Also the penis of male *L.scutulata* can be rapidly withdrawn. These differences, quite likely, have important adaptive value to each species.

PART 2: SPERMATOGENESIS

Introduction

The spermatogenic process has been studied extensively in prosobranch snails with the light microscope. Several excellent reviews of this subject have appeared in the literature during the past seventy years (Retzius, 1906; Wilson, 1925; Tuzet, 1930: Ankel, 1936; Nath, 1956; Franzen, 1955; 1970). However more recent studies on this group employing light and electron microscopy are in disagreement with these early workers on several points (Hanson et al., 1952; Gall, 1961; Walker and MacGregor, 1968; Garreau de Loubresse, 1971).

In the present study four of these points will be examined in detail for *Littorina sitkana* and *L. scutulata* and the results discussed in the light of previous work on prosobranchs and other animals. Additionally, a preliminary account of spermatogenesis and the origin of nurse cells (apyrene sperm?), at the electron microscope level, will be given. Specific points to be examined are as follows:

1. The formation and migration of the 'ring centriole' (Meves, 1903). This is the 'annulus' of vertebrate sperm.

2. The formation and fate of proximal and distal centrioles during spermiogenesis.

3. The formation of the acrosome.

4. The condensation of the nuclear chromatin.

To date none of these processes has been described in the genus Littorina at the electron microscope level, although most of the early light microscopists included one or more species of this group in their observations. In fact there are comparatively few fine structural studies of the spermatogenic process in molluscs as a whole.

Observations

A cross section through the testicular wall has the typical appearance described by Bloom and Fawcett (1968) for mammals. Spermatogonia divide mitotically several times before forming primary spermatocytes. These divide meiotically to form secondary spermatocytes which divide again to give rise to spermatids. The spermatids are formed in syncytial groups interconnected with each other by cytoplasmic bridges, and with the pseudopodia of nurse cells by desmosome-like junctions (PLATE 16B). The number of sperm in a clone, on average, is 256. Nurse cells are generally regarded as atypical (apyrene) sperm but in many ways they resemble the Sertoli, or sustentacular cells of the mammalian testis. Either apyrene sperm or nurse cells have been found in many prosobranch genera and their occurrence may be shown to be universal in this group. However, at present, it is uncertain whether or not, they are equivalent

structures.

As the sperm mature the intercellular bridges disappear and individuals become attached to the nurse cell by just the tip of the acrosome. Mature sperm clumps are eventually released into the vas deferens and pass from there to the seminal vesicles where they are stored.

SPERMATOGONIA

Spermatogonia measure 3-4um in diameter with a nucleus about 2.6um in diameter which contains a prominent nucleolus. The nucleus usually has a patchy appearance due to the arrangement of chromatin . Mitochondria of varying sizes are distributed throughout the cytoplasm together with one or two Golgi bodies, scattered endoplasmic reticulum, and numerous ribosomes. Sometimes one sees a very elongate mitochondrion measuring up to 2.1um in length. In some sections such mitochondria have hazy ends indicating that they may be even longer. Atwood (1974b) working with serial sections of echinoderm sperm has found that some mitochondria, which appear separate in individual sections, are in fact connected and form a single unit. Perhaps this also applies to Littorina. However there cannot be less than four mitochondria in the mature sperm because the pitch of the mitochondrial spiral could not be achieved by fewer than four units.

In the peripheral cytoplasm one occasionally finds

membrane-bound, electron dense, granules; lipid droplets; and clear vesicles. There are two centrioles, prior to replication, positioned at right angles to each other.

Early and late spermatogonia are illustrated in PLATE 8A, B.

SPERMATOCYTES

Spermatocytes are about 4um in diameter. The nucleus has enlarged to about 3.5um in diameter and typically the chromatin is evenly dispersed throughout the nucleoplasm. Synaptonemal complexes (chromosome cores) are visible in this stage (PLATE 8C). Other cell contents are similar to those for spermatogonia. Primary spermatocytes are shown in zygotene in PLATE 8C, and in late pachytene or early diplotene in PLATE 8D. A secondary spermatocyte is shown in PLATE 8E.

SPERMATIDS

These are grouped into stages A, B, C and D, based on the major changes that occur in their development.

Stage A (PLATE 8F)

The stage A spermatid is an approximately spherical cell about 3um in diameter with a nucleus measuring 1.75um across. The nuclear material has begun to condense at the periphery and so the centre is less dense.

Stage A spermatids are characterised by the thickening of the inner nuclear membrane in two areas, at the base and the apex of the nucleus. Garreau de Loubresse (1971) referred to the basal thickening as the 'differentiated zone', which he observed in detail in Nerita senegalensis. The differentiated zone is denser than the apical thickening of the nuclear membrane.

Both mitochondria and endoplasmic reticulum are scattered throughout the cytoplasm and many free ribosomes are visible. One or more Golgi bodies and several Golgi vesicles are found at the base of the nucleus.

The distal centriole, with perpendicular procentriole, has become attached to the plasma membrane prior to the invagination of the membrane towards the differentiated zone.

Stage B (PLATE 9A)

The differentiated zone has become indented and the distal centriole, bringing the plasma membrane with it, has come to lie in this depression. Endoplasmic reticulum is scattered in the cytoplasm together with numerous free ribosomes. The Golgi body has begun its migration to the apex of the nucleus and at this stage, usually one can see three types of vesicle produced from it. Small granular vesicles are budded off posteriorly; one large granular vesicle, usually with an oval shape, is produced anteriorly;

and two or three smaller, very dense vesicles, are produced alongside the oval granule in the anterior portion . The larger vesicle is the pro-acrosomal granule whereas the dense vesicles form the interstitial granule between acrosome and nucleus (PLATE 9B). The mitochondria have aggregated at the base of the nucleus and appear larger in diameter and spherical or oval in shape. The nucleus has become flattened and the chromatin aggregated at the periphery thus giving it a 'doughnut' shape.

Stage C (PLATE 9C)

The stage C spermatid is characterised by: a partially formed acrosome and adjacent Golgi body; scattered mixed endoplasmic reticulum; an oval shaped nucleus with visible condensation of chromatin inside it; the intranuclear canal, which is surrounded by the differentiated zone, has penetrated to the very tip of the nucleus where the basal body of the flagellum is situated; giant Nebenkerne(mitochondria) have fused in a single sheath around the flagellar shaft. The Nebenkerne are not yet twisted around the flagellum; the 'ring centriole' appears as two dense bands at the base of the Nebenkerne tube. The cytoplasm contains various products of degeneration including numerous clear vesicles, a few lysosome-like bodies and some ribosome material. The Golgi body continues to produce small granular vesicles from its cisternae. These may function as primary lysosomes in reducing the

cytoplasmic contents during maturation of the spermatid.

Stage D (PLATE 9D)

The stage D spermatid is characterised by its very elongate shape; a completed acrosome positioned anteriorly to the nucleus; nuclear elongation with condensed chromatin, in the form of plate-like lamellae, in the nucleoplasm; a distinct middle piece composed of four or five Nebenkerne arranged in a spiral around the flagellum; a Golgi complex in the cytoplasm lateral to the nucleus and middle piece; two dense bands of material at the base of the Nebenkerne, representing the ring centriole; an elongate tail, usually passing into the cytoplasm of an adjacent spermatid. The cytoplasm of the stage D spermatid contains fewer vesicles and ribosomes than in previous stages.

The characteristics of these four spermatid stages are summarised in PLATE 19 A-D.

1. Formation and Migration of the Ring Centriole (PLATES 9; 10)

In the stage B spermatid, a ring of dense material, joined to the distal centriole by nine spokes arranged in cartwheel fashion (PLATE 9E), becomes attached to the plasma membrane (PLATE 9F; 10A-D). In some sections the cartwheel arrangement resembles the satellite complex of Cnidaria (Szollosi, 1964; Summers, 1970; 1972a; Hinsch and Clark,

1973) and Echinodermata (Atwood, 1974a; Atwood et al. 1974). At this time a daughter procentriole is located perpendicular to the distal centriole at its anterior end.

Either by the downward flow of cytoplasm or perhaps by the upward migration of the distal centriole, as reported in <u>Nerita senegalensis</u> (Garreau de Loubresse, 1971), the centriolar complex comes to lie at the base of the nucleus close to the differentiated zone. The distal centriole begins to produce the flagellum posteriorly which pushes the invaginated plasma membrane back on itself creating a cylindrical vesicle, open at the posterior end, surrounding the flagellar shaft (PLATE 10D).

The cartwheel structure joining the dense ring to the distal centriole either breaks at nine points on its circumference, or degenerates, thus freeing the cylindrical vesicle with its attached ring. This dense ring is the structure that has been previously called the 'ring centriole' or 'annulus'. As the tail elongates further the invaginated plasma membrane is gradually everted and the mitochondria become aligned along the flagellar shaft. Their fusion results in the formation of a cylindrical tube composed of four or five giant Nebenkerne, enclosed in a single membranous sheath, which eventually become twisted around the flagellar shaft. The ring centriole comes to lie at the posterior end of the mitochondrial cylinder, where the tail forms (PLATE 10F). At this point a small

invagination of the plasma membrane creates a joint-like structure which is important in the following section on sperm motility.

Thus the 'ring centriole' forms from the distal centriole. In the mature sperm it appears as two dense bands on either side of the flagellum which implies either that it has replicated, or that it has become spiralled around the flagellum. Superficial sections in this region support the idea of a spiral structure (PLATE 10E).

2. The Formation and Fate of the Proximal and Distal Centrioles (PLATE 11)

At the time when the distal centriole first comes to lie at the base of the nucleus, the daughter procentriole lies perpendicular to its anterior end (PLATE 11A). In *Littorina* the procentriole does not disintegrate as suggested for other molluscs (Gall, 1961; Walker and MacGregor, 1968; Garreau de Loubresse, 1971), nor does it become a mature proximal centriole as might be expected. Instead it rotates through 90° and aligns itself with the apical end of the distal centriole (PLATE 11B, C). Fusion occurs and a dense plate is formed (to which the distal centriole contributes), below the procentriole (PLATE 11E). Since the procentriole occupies the basal portion of the flagellum it will be referred to as the 'basal body' of the sperm. The nine outer fibres between the basal body and

distal centriole elongate during spermiogenesis so that the structures are finally separated by a distance of 0.26um in the mature sperm. The nine outer fibres are therefore continuous between the centrioles but the central fibres originate in the distal centriole.

3. <u>The Formation of the Acrosome</u> (PLATES 11; 12)

The Golgi body migrates to the anterior end of the nucleus together with the interstitial granules and the proacrosomal granule. The interstitial granules appear to fuse (although no direct observations of this have been made), and the single dense granule comes to lie over the nucleus, directly above the basal body of the flagellum. The proacrosomal granule, which is now in the form of a hemisphere indented at the base, positions itself over the interstitial granule (PLATE 12A).

The acrosome cone begins to differentiate from the base of the hemisphere, apically and as it does so the hemisphere becomes more cone-shaped (PLATE 12B, C). A dense matrix is produced within the basal indentation of the hemisphere and sometimes one can see tiny tubular structures within this substance. The matrix eventually forms the acrosomal rod which is supported internally by four or five fine tubules.

A dense substance is also produced within the membranes of the hemisphere and this gradually coalesces to form the cone-shaped acrosomal granule. During this time the

interstitial granule becomes flattened into a thin disk (PLATE 12D). This may be due partly to the pressure exerted on it by the acrosome which at this stage is surrounded by microtubules (PLATE 12F). These microtubules may also be responsible for causing the acrosomal elements to be formed into a cone-shape. The microtubules disappear in the mature sperm.

When the acrosome cone is complete, the Golgi body migrates posteriorly (PLATE 9D). After this it is said to be sloughed off (Nath, 1956) but this has not been observed in *Littorina*.

4. Condensation of the Nuclear Chromatin (PLATES 13; 14)

Condensation of chromatin begins in the early spermatid from the periphery of the nucleus towards the centre. This produces the characteristic 'doughnut' appearance of the stage B spermatid. Later in this stage the chromatin appears granular in form (PLATE 14A). It is not until stage C that one sees condensation of granules into fibrous strands (PLATE 13B), which slowly aggregate to form lamellar plates in the stage D spermatid (PLATE 13C). Condensation begins at the centre and at the periphery , and gradually works its way through all the nucleus. A unilateral arrangement of microtubules is evident during the last part of this process (PLATE 14E). These microtubules appear to spiral around the nucleus. Rebhun (1957), observed a similar spiralling of

microtubules in <u>Otala lactea</u>, a pulmonate snail.

The formation of the lamellar plates is characterised by small tubule-like arrangements of chromatin adjacent to the inner nuclear membrane (PLATE 13D). The leaflets, or lamellae, are attached to these tubules of chromatin and initially radiate out evenly from the centre, through the nucleoplasm . They remain this way in the anterior portion (PLATE 13F) but in the posterior regions, as condensation progresses, they become folded back on themselves (PLATE 14B) and twisted in a gentle spiral around the flagellar shaft (PLATE 14D). Eventually the lamellae appear as a series of concentric rings of dense chromatin surrounding the flagellum with gaps appearing only in a few places (PLATE 14A, B). Prior to this stage one sometimes sees single strands, which appear circular in cross-section, indicating the presence of fibres (PLATES 13E; 14B, C). These later coalesce with the lamellar plates to form a homogeneously dense nuclear tube (PLATE 14E). Condensation appears to begin anteriorly and gradually works its way posteriorly over the entire nucleus (PLATE 14A).

<u>Preliminary observations on the origin and formation of nurse cells (apyrene sperm) and their association with eupyrene sperm</u> (PLATES 15-18)

Nurse cells originate in the testicular wall from diploid cells which undergo a special type of reductional division that leaves the nurse cell nucleus with half the

volume of a normal diploid cell. This has been described in detail by Koshman and Serra (1967) and Serra and Koshman (1967) for two pulmonate snails <u>Cepaea nemoralis</u> and <u>Helix aspersa</u> and the type of reduction division has been termed "spireme kariodieresis".

Nurse cells send out pseudopodia which interdigitate with developing sperm cells and other nurse cells (PLATES 15; 16A, B). These pseudopodia contain mitochondria, lipid droplets, endoplasmic reticulum, free ribosomes, glycogen-like particles and clear vesicles. All these constituents are present in the cytoplasm of the nurse cell plus several lysosomes. Secondary lysosomes are commonly found in the process of digesting part of the cytoplasm or, later on, the nucleus of the nurse cell (PLATE 16C).

Eventually the nurse cell is released from the testicular wall at which time its cytoplasm contains large quantities of granular endoplasmic reticulum within which fine grain secretions are produced (PLATE 17A). Many mitochondria are found in the cytoplasm. The nucleus is indented on one side and within this indentation is an area of pale-staining secretory vesicles (PLATE 17A). The function of these vesicles is not known. Serra and Koshman (1967) mention the formation of spaces or lacunae without chromosomes in the nucleus, which they believe "suggests that some process of interaction between chromosomes and the nucleoplasm is going on while successive endomitoses are

occurring". Eupyrene sperm become attached to the nurse cell membranes at least as early as the spermatid stage. Serra and Koshman have found that in pulmonates the sperm do not attach to the nurse cell, unless it becomes diploid by fusing with a second nurse cell. They suggest that polyhaploid nurse cells are immature and secretory in function whereas polydiploid nurse cells are mature and give nourishment to the sperm.

The plasma membranes of the eupyrene sperm form desmosome-like junctions with the membranes of the nurse cell pseudopodia (PLATE 16B, Inset). As the nurse cell matures the pseudopodia are gradually withdrawn so that the attached sperm form a cluster on one side (PLATE 16D).

The transfer of substances from nurse cell to eupyrene sperm in Littorina has not been observed though, quite likely, it occurs. However the mature sperm contains numerous glycogen granules in the tail region (Anderson, 1972) and these are not present until a late stage (beyond spermatid stage D) in development. Possibly nurse cells in some way contribute to the formation of these glycogen granules.

As the nurse cell matures further it becomes spherical in shape and the nucleus disintegrates (PLATES 17B; 18A). The products of breakdown of nucleus and cytoplasm are large membrane-bound lipid and mucopolysaccharide droplets, which can be seen forming in PLATES 17A, B; 18A.

Mature sperm are attached to the nurse cell only by the tips of their acrosomes.

Discussion

Formation and Fate of 'Ring Centriole'

It is interesting that some light microscopists consistently observed the posterior migration of a small, ringed structure encircling the flagellar shaft (which they interpreted as the distal centriole), during the formation and alignment of the mitochondrial Nebenkerne. They located the final position of the 'ring centriole' at the junction between tail and middle piece (Nath, 1956; Franzen, 1955; 1970). However, many electron microscopists found that the distal centriole produces the flagellum and remains adjacent to the nucleus which meant that the ring centriole had to be something else, so it was deemed "a cytoplasmic secretion", or as one author mused, "an optical illusion" and it was forgotten. Fawcett renewed interest in the structure when he located it in mammals and followed its development in connection with the chromatoid body, calling it the 'annulus' (Fawcett, 1958; 1965; 1971). The 'annulus' (ring centriole), had been previously found at the light microscope level in mammals, birds, fish, turtles, amphibians and molluscs.

Thompson (1966) described an annulus that remains

attached to the distal centriole in the mature sperm of *Archidoris pseudoargus*, a nudibranch gastropod . The manner in which the ring centriole forms in *Littorina* has not been previously reported. It is easy to understand now, why the early light microscopists confused it with the distal centriole, since it is formed from this structure. The implication is that a similar process may also occur in vertebrate sperm and other invertebrate sperm, and may account for the presence of structures such as "pericentriolar primary processes" found at the base of the distal centriole in *Metridium dianthus* (Hinsch and Clark, 1973). These "processes" resemble the cartwheel structure in *Littorina* that gives rise to the ring centriole .

Formation and Fate of the Proximal and Distal Centrioles

Most invertebrate sperm have two centrioles, proximal and distal, located adjacent to the nucleus. However, many gastropods are reported as having a single, distal, centriole which produces the flagellum, the proximal centriole either having failed to replicate during meiosis, or disintegrated at the spermatid stage (Hanson et al., 1952; Gall, 1961; Walker and MacGregor, 1968; Walker, 1970; Garreau de Loubresse, 1971). Gall does mention that a "daughter procentriole" is found in the spermatid of *Viviparus contectoides*, positioned at right angles to the anterior end of the distal centriole . Yasuzumi and Tanaka (1958) describe two centrioles at the anterior end of the

flagellum of the snail Cipangulopaludina malleata but Gall says that this observation is "certainly in error" and that their proximal centriole "is apparently a portion of the nuclear envelope". Thompson (1966) describes two perpendicular centrioles in Archidoris pseudoargus , which he illustrates clearly as lying at the base of the nucleus. The appearance of the centriolar complex in the oligochaete Enchytraeus albidus (Reger, 1967) closely resembles that of Littorina. Reger, perhaps quite rightly, considers the small anterior centriole as the true proximal centriole.

Rosati et al. (1970) report an almost identical centriolar complex to that of Littorina, in the spider Pholcus phalangoides. Their description follows; "a small channel is invaginated into the still spherical nucleus, and it is occupied by a centriole structure that in longitudinal section seems to result from two aligned units ie. two coaxial parallel centrioles. In this region it bears an absolutely unusual pattern of fibrils." They conclude; "This structure, with some insignificant variations, remains the same in other families of spiders." It is interesting that in other respects spider sperm resemble that of the cephalopod Octopus bimaculatus described by Longo and Anderson (1970).

In the sperm of several different genera of Cnidaria two centrioles become aligned, the proximal being smaller than the distal (Summers, 1972a). universal in this group as

in Pennaria (Summers, 1970), Hydra (Burnett et al., 1966); Campanularia (Lunger, 1971) and Nausithoe (Afzelius and Franzen, 1971), the proximal centriole lies at an angle of 45-90° to the distal centriole. This is also apparent in some sections of Littorina spermatids (PLATE 11D) but micrographs of mature sperm show that the final position of the procentriole is coaxial with the distal centriole (PLATES 11E; 21I). Another interesting point is that Summers (1972a) says that the central tubules arise in the distal centriole in Eudendrium ramosum which is the same as in Littorina.

My observations on Littorina support the existence of a daughter procentriole in the spermatid stage which may be a modified proximal centriole. This structure becomes attached to the anterior end of the distal centriole after an alignment process that involves 90° rotation and thus becomes the basal body of the flagellum. The distal centriole may contribute to the production of this basal body but it begins as a separate entity, the procentriole. This process has not been previously described in molluscs although Gall mentions that "the procentriole may remain as a germinal centre in the mature (distal) centriole" and "one end (of the distal centriole) is itself derived from a procentriole".

Acrosome Formation

Meves (1899), working on mammals, and Montgomery (1911), working on insects both described the formation of the acrosome from the 'idiosome', or 'sphere', which was later recognised as the Golgi apparatus (Bowen, 1922a; 1924). According to Gupta (1955) the development of the acrosome is from an acrosomal granule positioned on top of the nucleus which passes through a triangular stage with deposition of material beginning from the apex and passing distally. In *Littorina* the acrosome granule also appears conical and some dense material is seen on the apical surface of it prior to acrosome formation (PLATE 11F). However the deposition of the acrosome itself proceeds in the granule from the base, apically (PLATE 12B, C). The formation of the acrosome from the Golgi apparatus was reviewed by Nath (1956) in a wide variety of animals including amphibians, molluscs and mammals. Garreau de Loubresse (1971) reports an exception to this general rule in the pulmonate *Nerita senegalensis* in which, he says, the acrosome forms without assistance from the Golgi complex.

The descriptions of acrosome formation by the early light microscopists were very accurate. At least one Golgi body (and perhaps more) participates in acrosome formation though it has not been possible to verify the existence of the dictyosome 'triangle' or 'rhombus', to which Nath referred (1956). This is probably due to the orientation of

the block and should not be taken as a statement against their existence.

Acrosome development in _Littorina_ is similar to that for: the house cricket, _Acheta domestica_ (Kaye, 1962); the polychaete, _Spirorbis morchi_ (Potswald, 1967); the prosobranch, _Nucella lapillus_ (Walker and MacGregor, 1968); and the cirripedes, _Balanus balanus_ and _B. perforatus_ (Munn and Barnes, 1970b). Walker and MacGregor called the dense granules beneath the acrosome "the interstitial membrane" and said that it derived from "diffuse material at the base of the pro-acrosome". However this description does not fit the situation in _Littorina_, in which the "interstitial membrane" derives from a dense Golgi vesicle. Thus, in this account, the term 'interstitial granule' has replaced "interstitial membrane".

Condensation of Nuclear Chromatin

Condensation of nuclear material in prosobranchs has been reported by: Grasse et al., (1956); Rebhun, (1957); Kaye, (1958b; 1969); and Idelman (1961). However, in spite of similar micrographs, their descriptions differ as to the structure of the elements in the nucleus at the different stages.

Rebhun states that the condensation process involves the formation of lamellar plates, whereas Grasse et al. and Idelman describe the development of fibres. Kaye (1969)

reviewed the process and found that usually the formation of lamellar plates predominates but that this is sometimes preceded by an initial formation of fibrous strands. He reports that in some molluscs the entire process involves condensation of fibres though this is not true in the animals described by Grasse et al. . Rebhun's description is extremely clear and he also provides an accurate three-dimensional diagram that very much resembles the situation found in the later stages of the condensation process in *Littorina*. He also mentions that radial formation of chromatin occurs close to the apex of the nucleus as noted in *Littorina*. However he did not record the presence of fibres in both early and late stages of development as are clearly seen in *Littorina* (PLATE 13E; 14B, C). These fibres eventually coalesce with the lamellar plates. Similar processes of condensation have been reported in other phyla, such as the annelids (Potswald, 1967; Reger, 1967).

Nurse Cells and Eupyrene Sperm (Spermatozeugmata)

The mature sperm are firmly attached to the nurse cell by the tips of their acrosomes. On several occasions numerous small blebs have been observed covering the apical end of the acrosome. Viewed at high magnifications they are indistinct (PLATE 18B) but bear some resemblance to the stalked particles identified on cirripede acrosomes by negative-staining procedures (Munn and Barnes, 1970a). The function of the blebs is unknown but since the sperm are

attached to the nurse cells only in this region, they may be involved in adhesion. Evidence in support of this idea was obtained from electron micrographs at very high magnifications, in which one can see septa between the plasma membranes of spermatid and nurse cell (PLATE 18C).

Goldschmidt (1916) believed that "the apyrene sperm is a functionless reaction product necessitated by the physico-chemical properties of the (normal) sperm cell on which abnormal surroundings act; a reaction produced by abnormal conditions". This does not seem to be true for molluscs because in many species the apyrene sperm serve a definite function in transporting the eupyrene sperm. In cases where this has doubtful application, such as in Littorina, the nurse cell may provide nourishment for the sperm both when it is intact in the male, and when it has degenerated in the female. Additionally, the products of nurse cell destruction in the bursa furnish this organ with nutrients. Possibly there are other functions which have not been observed, or envisaged yet.

In some species of Littorina the nurse cell is more complex. Reinke (1912) reports that the nurse cell of L.angulifera is elongate and encloses a dense cytoplasmic rod to one end of which, the sperm are attached. He believes that the cytoplasmic rod may derive from a centrosome (centriole), which is not present in the nurse cells of other Littorina. It would be interesting to review the

phylogeny of the Littorinidae , keeping observations such as these, in mind. In this regard, the nurse cell might represent either a primitive form of apyrene sperm (compared to the bizarre atypical sperm of other species), or an advanced form that has become simplified. The latter is less likely since the Littorinidae are considered to be a fairly primitive family of mesogastropods.

PART 3: THE FINE STRUCTURE AND MOTILITY OF SPERM

Introduction

Spermatozoa of marine invertebrates which utilise external fertilisation, usually have a large spherical head with a long flagellum and are considered to be primitive. However, species with internal fertilisation usually have modified filamentous sperm. Franzen (1955, 1956, 1970) suggested that the morphology of invertebrate spermatozoa is related to the biology of fertilisation. This correlation was shown to be particularly evident in the phyla Nemertini, Annelida and Mollusca. Franzen believes that the phenomenon has a phylogenetic basis such that, during molluscan evolution, the change from a primitive sperm to a modified filamentous one coincided with the adoption of internal fertilisation in place of free-spawning. One of the important differences in these two modes of fertilisation is the change in viscosity of the medium in which the sperm move. The medium of the female genital tract is more viscous than sea water, due to the presence of mucus and other secretions. Coakley and Holwill (1971) have shown that as the viscosity of the medium increases the presence of a head becomes a limiting factor. However, at reduced viscosities within a definite size range, a head is an advantage as it tends to minimise both non-uniformity of the beating flagellum and its detrimental effects on the propulsive velocity and power expenditure. It seems then that one of

the reasons for the retention of the primitive type of sperm is that it is specifically adapted for efficient swimming in a sea water medium, where viscous forces are not sufficiently high to outweigh the advantages of retaining a head. The filamentous shape is usually inefficient for swimming in sea water because the wavelength and amplitude of undulation increase to such an extent that the sperm fails to form a complete wavelength along its body, and so remains stationary in spite of producing rhythmical undulations (Gray, 1951).

Owing to the small Reynold's number, (which is a numerical expression of the ratio: stress due to inertia/ stress due to viscosity), characteristic of microscopic oscillators such as spermatozoa, the inertial forces are negligible compared to the viscous forces acting on the body. The viscosity of the medium therefore influences both the waveform of the moving sperm and the efficiency of the propulsive mechanism (Gray, 1953). Littorina sperm are exceptional because they are capable of rapid propulsion in both viscous and sea water media, and yet are filamentous.

The functional morphology of the mature sperm of Littorina has not been previously studied and, in general, relatively little information has been published on the ultrastructure of mollusc sperm. In cases which have been studied with the electron microscope, the sperm of Crassostrea virginica (Daniels et al., 1971; Galtsoff and

Philpott, 1960); *Spisula solidissima* (Longo and Anderson, 1969a); *Mytilus edulis* (Longo and Dornfield, 1967) , demonstrate the primitive type, whereas the sperm of *Opalia crenimarginata* (Bulnheim, 1962b); *Nucella lapillus* (Walker and MacGregor, 1968); *Octopus bimaculatus* (Longo and Anderson, 1970); *Viviparus viviparus* (Hanson, Randall and Bayley, 1952); *Helix aspersa* (Anderson and Personne, 1969a); *Otala lactea* (Rebhun, 1957); and *Littorina scutulata* exhibit the modified, filiform shape. This section describes the fine structure and motility of the sperm of *Littorina scutulata* (the sperm of *L.sitkana* is virtually identical).

Observations on Sperm Fine Structure

The spermatozoan of Littorina scutulata has a filiform shape and is about 50um in length. For descriptive purposes the sperm will be divided into four regions: Acrosomal, nuclear, mitochondrial and tail.

Acrosomal Region

The acrosome is a cone-like structure 0.7um long, bounded by an acrosomal membrane. At its base there is an invagination, in which lies the acrosomal rod that is supported by fine tubules. The plasma membrane of the sperm overlies the acrosomal membrane and with it forms the acrosomal vesicle at the very tip of the sperm. Between the base of the acrosome and the nuclear envelope is the dense interstitial granule (PLATE 20A). There are no microtubules present around the acrosome of the mature sperm.

Nuclear Region

The nucleus is an elongate, cylindrical structure, measuring 23um in length, with a tip diameter of 0.2um and a basal diameter of 0.4um . The nuclear material surrounds the flagellar shaft (PLATE 20B, C), which extends the entire length of the sperm excluding the acrosomal region. The width of the nuclear material at the tip is 13mu and at the base 98mu . A double-layered nuclear envelope surrounds the apical third of the nucleus but does not extend to the basal

portion (PLATE 20C). Thus for two-thirds of its length the nucleoplasm is in contact with the cell cytoplasm. At the tip of the nucleus the nuclear envelope is invaginated a distance of about 0.2um outside the flagellar shaft (PLATE 20A). It is a characteristic of this sperm to find membranous vesicles in the cytoplasm outside the nucleus, beneath the plasma membrane (PLATE 20C). These vesicles are possibly redundant portions of the nuclear envelope (Potswald, 1967; Summers, 1970). One such vesicle is consistently found in the basal two-thirds of the nucleus, inside the nuclear tube, lying adjacent to the flagellar shaft and always overlying flagellar fibres 7, 8 and 9 (using the system of Afzelius, 1959) (PLATE 20B). In this region a single microtubule lies adjacent to the external surface of the nucleus, always located between flagellar fibres 3 and 4 (Summers, 1972a) (PLATE 21B). Where this microtubule ends in the apical third of the nucleus, a second microtubule begins adjacent to the internal surface of the nucleus, between flagellar fibres 5 and 6 (PLATE 21A). This second microtubule terminates at the "basal body" and can be seen in cross sections up to this point (PLATE 21A). The diameter of both microtubules is about 12mu . The positions of the vesicle and microtubules appear to be constant in all sections of sperm but their formation has not been studied.

At the anterior tip of the nucleus the flagellar shaft terminates at the basal body which is probably a modified

proximal centriole as described in the previous section. It is atypical because it does not comprise nine sets of triplet fibres. It is 0.15um long and is embedded in an electron dense material, which appears as a dense circular structure (basal plate) in cross section (PLATE 21E).

The distal centriole, which is 0.46um long and 0.18um wide, is located at a distance of 0.26um from the basal body (PLATE 21I). It is in the distal centriole that the two central fibres arise (PLATE 21I).

Sections through the distal centriole do not show the characteristic arrangement of nine peripheral triplet fibres, as was reported for Nucella lapillus (Walker and MacGregor, 1968). Instead there is a ring of nine flagellar doublets interconnected by a complex array of fibres (PLATE 21F). The nine doublets are continuous with the basal body but the pattern of connecting strands changes more proximally (PLATE 21F, G, H). Just prior to the formation of the distal centriole each A fibre of the doublets bears two "arms". In sections through the distal centriole the inner arm disappears and is replaced by a continuous strand that interconnects all the peripheral doublets (PLATE 21G). This strand is visible in sections above the distal centriole (PLATE 21H) and in the region of the basal body it appears to be continuous with the material forming the outer doublet fibres (PLATE 21C, D). In sections just posterior to the distal centriole, the outer arm of each A subfibre appears

to form a small hook or tubule (PLATE 22A). Similar structures were observed in mussel-gill cilia (Warner and Satir, 1973). The hook (or tubule) formed by the A subfibre of doublet 6 appears to give rise to the supernumerary tubule adjacent to the nucleus between doublets 5 and 6 (PLATE 22A).

During spermiogenesis the nuclear material condenses in a series of lamellae, spiralled around the flagellar shaft (PLATE 23A). The spiral is in a clockwise direction from head to tail in the mature sperm. These fibres are constrained under considerable tension for when the sperm are placed in 2x concentrated sea water to disperse the cell and nuclear membranes, the nuclear portion twists into a number of coils. These phenomena have been previously observed in Littorina by Retzius (1912), and in Nucella lapillus by Walker (1970).

Mitochondrial Region

The mitochondrial region is characterised by a spiral arrangement of four, or five, Nebenkerne forming a tube 14.5um in length and 0.53um in diameter, surrounding the flagellar shaft (PLATE 23B). At the proximal end, the mitochondrial tube is separated from the tapered base of the nucleus by a distance of about 23mu . The mitochondrial Nebenkerne are each about 0.1um in width and are enclosed in a single sheath formed from the fusion of the individual

outer mitochondrial membranes during spermiogenesis. A short distance behind the proximal end of this region, the mitochondrial sheath fuses with the plasma membrane creating one or more pores about 75mu in diameter (PLATE 22B). This is unusual as it means that the mitochondria are in direct contact with the external environment.

Tail Region

The tail is 11um long and may be divided into two parts: (1) The proximal portion, which is 0.6um in diameter and 8um in length, contains numerous glycogen granules overlying the flagellar shaft (Bulnheim, 1962a; Anderson and Personne, 1969b; Anderson, 1972). There are no internal membranes in this region and in some sections it appears that the glycogen granules are arranged in groups opposite each pair of peripheral doublets (PLATE 23D). (2) The distal portion does not contain glycogen granules, is 0.34um in diameter and 3um in length. In this portion the outer doublets terminate just before the central fibres at the tapering end of the tail (PLATE 23C).

At the junction between tail and mitochondrial regions there is a "joint" which is clearly visible on scanning electron micrographs (PLATE 24A) and also in longitudinal sections (PLATE 24B). This joint is supported by the ring centriole (annulus), which is visible as two densely staining bands of material each 0.1um in width, which may

form one continuous spiral outside the flagellar shaft (PLATE 23D).

In surface sections through the junctions between tail and mitochondrial regions, one often sees that the peripheral doublets are spiralled (PLATE 24B). Additionally, the tail may be held at right angles to the main axis of the flagellar shaft, the bend occurring at the "joint", previously mentioned (PLATE 24C).

Observations on Sperm Motility

In both sea water and oviductal fluid the first phase of the propulsion mechanism involves an anti-clockwise undulation (looking from tail toward acrosome), during which the tail portion rotates at a faster rate than the acrosomal end causing a visible twisting of the flagellum (PLATE 27, frames 1-37). During this phase, helical waves pass from tail to acrosome. In the second phase the sperm shortens by bending in the mitochondrial region, accompanied by supercoiling in the nuclear portion (PLATE 27, frames 35-37) The sperm begins to uncoil in a clockwise direction at a rapidly increasing frequency with 3-dimensional waves passing from acrosome to tail (PLATE 27, frames 38-57). Once the sperm begins this phase, forward propulsion is initiated; prior to this the sperm is stationary.

As the sperm accelerates, the frequency of its rotation increases causing a series of changes in the visible

waveform until, in the final phase, a stable state is reached. At 12 revs./sec. a binodal wave is set up (PLATE 27, frames 48-58). As the frequency increases the binodal wave becomes unstable and passes through a short period of turbulence (PLATE 27, frames 60-63) before developing an asymmetric trinodal wave (PLATE 27, frames 64-69). During this phase the average frequency of rotation is 18 revs./sec The waveform again becomes unstable and there is a second period of turbulence (PLATE 27, frame 70) before the symmetric trinodal wave is formed (PLATE 27, frames 71-73). In this conformation rotational frequency and propulsive velocity are maximal at 24 revs./sec. and 185um/sec. respectively. The tail beats obliquely to the main flagellar axis causing node 3 to form at the tail joint. Node 1 advances with the tip of the acrosome and node 2 forms in the nuclear region. Both the amplitude and wavelength between nodes 2 and 3 are greater than between 1 and 2, creating a 3-dimensional waveform that resembles a tapered screw thread (PLATE 26A). The maximum velocity is attained in sea water, oviductal fluid and 12% Protoslo solution. Solutions of higher viscosity tend to slow the sperm and at 30% Protoslo most sperm are unable to initiate propulsion. Independent beating of the tail has been observed in situations where the anterior portion of the sperm has become attached to the surface of coverslip or slide and the tail beats vigorously in an attempt to free it (PLATE 26B). Sometimes the frequency of the tail beat does not increase

and the binodal waveform is maintained. The sperm therefore swims at a reduced velocity. If the frequency of tail beating increases the waveform changes to the stable trinodal wave and maximum velocity is attained. The slower form of movement occurs frequently when the water is very cold (5 °C) , when the sperm have been active for some time, or when the sperm are removed from a snail that was killed a day or so previously. However, fresh sperm usually swim at maximum velocity in the trinodal configuration. Littorina sperm may also swim slowly in a tail-first direction by producing 3-dimensional bending waves from the tail toward the acrosome. This movement has not been studied in any detail and it is very slow (18um/sec) compared to forward propulsion (185um/sec), but it is important because it means that waves can be propagated in either direction along the sperm.

Nurse Cell Motility

It is well known that in many prosobranchs bizarre, atypical (apyrene) spermatozoa are found in addition to typical (eupyrene) sperm that fertilise the eggs (von Siebold, 1836; Brock, 1887; Meves, 1903; Ankel, 1930; Linke, 1933; Graham, 1954; Fretter and Graham, 1962; Bulnheim, 1962a,b; Dembski, 1968). In many cases these atypical sperm, with their attached sperm clump, can propel themselves through the water rapidly by their own undulations. An example of this are the spermatatozeugmata of Scala and

Janthina (Ankel, 1926). In Littorina the spherical nurse cells are incapable of propelling themselves but with the aid of the synchronised beating of the attached sperm clump, they are driven through the water rapidly (150 - 250um/sec) in clockwise spirals. This was first observed by Reinke (1912) in three species of Littorina ; L.angulifera, L.rudis and L.nebulosa (this species is unknown to me and does not appear in subsequent reviews).

The swimming of these spermatatozeugmatata is only short-lived, because the sperm begin to beat out of phase with each other and the clump spreads. Soon afterwards individual sperm free themselves and shoot off in all directions.

Discussion

The functional morphology of the spermatozoan of Littorina scutulata will be compared to that of previously described mollusc sperm and sperm from other phyla, on a regional basis. The fine structure of Littorina sperm is summarised in PLATE 25.

SPERM FINE STRUCTURE

Acrosomal Region

The acrosome of Littorina sperm is very similar to that of Nucella lapillus (Walker and MacGregor, 1968). The "ragged membrane" found in Nucella is also apparent in the

mature sperm of <u>Littorina</u>, but in this snail it is not a membrane. Possibly it is the remains of a dense substance produced by the acrosome during its formation (PLATE 11F; 12B, C) or it is the remnants of the acrosome microtubules (PLATE 12F).

While the sperm are attached to the nurse cell the acrosomes are subjected to considerable bending stresses owing to the active movements of the sperm flagellum, making it disadvantageous to have a rigid acrosome. Micrographs support this idea as the acrosome is often hook-shaped (PLATE 23E) or slightly compressed (PLATE 20A).

<u>Nuclear Region</u>

It is interesting to note that the evolution of a typical centriole to a modified basal body coincides with the development of a more elaborate and complex mitochondrial sheath (Andre, 1962; Fawcett and Ito, 1965). Examples of modified basal bodies, such as may exist in <u>Littorina</u>, are found in several other phyla (Fawcett, 1958, 1962; Sotelo and Trujillo-Cenoz, 1958). These authors presume that the basal bodies retain their function as the kinetic centre during motility (Anderson and Personne, 1967). However some experiments indicate that the true site of initiation of flagellar activity may be elsewhere, perhaps in the central fibres, or the arm of peripheral subfibre A, or between outer and central fibres (Anderson

and Personne, 1970a; Nelson et al., 1970a), or anywhere along the flagellum (Gibbons, 1972). In Littorina sperm waves can be propagated in either direction along the flagellum and the tail can beat independently. This observation has been reported previously by Holwill (1965) and appeared in reviews by Brokaw (1972) and Warner (1973). It has also been shown that if bending waves can be propagated along a flagellum with constant amplitude, in a viscous medium, they must be maintained by active processes distributed along the flagellum (Gray, 1955; Brokaw, 1972). Brokaw (1972) included information that suggested a single control centre for normal initiation of the beat cycle, in bull sperm. However this may not apply to Littorina since there appear to be at least two sites of beat initiation, at opposite ends of the sperm. It may be significant that the ring centriole, which derives from the distal centriole, occupies one of these sites, that is, the tail joint. Its importance in the motile mechanism cannot be understood without further experimentation but this need not be confined to species of Littorina since the ring centriole (annulus) is present in the sperm of; mammals, birds, fish, turtles, amphibia and many other gastropods.

Mitochondrial Region

The plasma membrane of the sperm appears to be thicker than other sperm membranes such as those of the mitochondria (PLATE 23C). The outer leaflet of the plasmalemma often

appears hazy in section, as if covered by some poorly-preserved, extraneous material. Bennett (1963) referred to an extraneous polysaccharide-rich coat on cell surfaces as the "glycocalyx", which is thought to be a dynamic surface coat functioning in a variety of physiological roles. Anderson (1968) found ruthenium red positive deposits on the surface plasmalemma of the head, middle piece and tail of the mature spermatozoa of sea urchins, indicating the presence of acid polysaccharides and related compounds. In the same paper Anderson reviewed the possible roles of this coat in the fertilisation process, suggesting the presence of enzymes and antigens on the surface of the plasmalemma.

The presence of pores in the mitochondrial region, formed by the fusion of the plasmalemma with the outer mitochondrial membrane, is interesting because it means that this region is in direct contact with the external environment. One possible function of these pores is the direct diffusion of substances (possibly oxygen) to the mitochondria, which is important in the situation, such as exists in the seminal receptacle, where numerous actively beating sperm are packed into a comparatively small, confined space. Another possible function is the more rapid reception of chemical substances such as might be produced by the egg membranes. The reception of such substances in the most active region of ATP synthesis and translocation (Anderson and Personne, 1969b) would be advantageous for a

rapid response by the sperm.

The spiralling of peripheral doublets that occurs at the junction between mitochondrial and tail regions is important because it implies localised bending of the flagellum, which correlates with light microscope observations of the independent tail beat. The 'joint' at the tail region is much more pronounced than the junction between mitochondrial and nuclear regions. One function of the ring centriole at this joint may be to prevent constriction of the flagellar shaft during the perpendicular phase of the tail beat cycle, since sections in the tail region during this phase of the beat show an unaltered configuration of flagellar fibres (PLATE 24D).

SPERM MOTILITY

Based on the above observations, the following hypothesis is proposed to explain rapid propulsion of Littorina sperm:

During the initial stages, torsion is produced in the flagellum by anti-clockwise rotation of the tail at the 'joint' (looking from tail toward acrosome). Owing to the anti-clockwise coiling of constrained nuclear elements and the clockwise coiling of relatively unconstrained mitochondrial elements, torsion is developed predominantly in the nuclear region of the sperm. When the torque is maximal the nuclear region is super-coiled but the rest of

the sperm is not. Contraction of flagellar fibres bends the sperm in the flexible mitochondrial region while the tail begins rotating in a clockwise direction. The release of torsion from the nuclear portion sends waves along the flagellum from acrosome to tail as the sperm rotates at a rapidly increasing frequency. Owing to the structural modifications in the sperm, particularly the tail joint and the mitochondrial-nuclear junction, a specific 3-dimensional waveform (the trinodal wave) is set up in the flagellum. The trinodal waveform enables the tail to act like a propeller, and the sperm to screw itself through the medium, by taking advantage of external, viscous forces. These forces would tend to prevent rotation of the flagellar axis in a clockwise direction and thus maintains an amount of torsion in the sperm, necessary for this waveform to exist.

Helical arrangements of structural elements in both nuclear and mitochondrial regions are of widespread occurrence among filamentous sperm from many different invertebrate and vertebrate groups, including: GASTROPODA (Franzen, 1955, 1970; Grasse et al., 1956; Yasuzumi and Tanaka, 1958; Gall, 1961; Anderson and Personne, 1967; Walker and MacGregor, 1968; Walker, 1970; Anderson and Personne, 1970b; Thompson and Bebbington, 1970); CEPHALOPODA (Franzen, 1966; Longo and Anderson, 1970); INSECTA (Phillips,1969); OSTRACODA (Reger,1970); SQUAMATA and CHELONIA (Furieri,1970); MAMMALIA (Fawcett,1965); to name but a few. The idea that constrained helical elements are

important in generating a torque for initiating propulsion in Littorina sperm may have general application to all filamentous sperm. Thompson (1966) noted that the sperm of Archidoris pseudoargus swim rapidly in a helical path and he attributed this to the arrangement of spiralled elements in the sperm. He also observed a decrease in amplitude of the 'optical envelope' with increasing speed and that semi-active sperm swim with greater amplitude. These observations correlate well with the above description of swimming Littorina sperm.

In conclusion, primitive sperm swim inefficiently in viscous media whereas the filamentous sperm of Littorina, and possibly of many other marine organisms, swim rapidly in media of widely ranging viscosity. Copulation success is high in Littorina and so the usefulness of this adapability is not obvious, although individual sperm set free in sea water are capable of swimming to a neighbouring female. However, it may be important to some closely related prosobranchs such as Turritella communis (Bataillon, 1921; Idelman, 1961) and Bittium reticulatum (Franzen, 1955) which lack a penis and do not have any obvious means of sperm transference from male to female snail, other than inherent sperm motility. Ciliary feeding currents of these snails may help in drawing the sperm into the mantle cavity.

PART 4: SPERM RESORPTION

Introduction

Sperm resorption by phagocytosis may occur at several sites in the male and female reproductive systems of gastropods: In stenoglossans it has been observed in the ingesting gland and seminal vesicles (Fretter, 1941; Ponder, 1972) and in the seminal receptacle (Fretter, 1941); in pulmonates it was reported in the seminal vesicles (de Jong Brink, 1969: Joosse et al., 1968; Breucker, 1964); and in mesogastropods it has been observed in the seminal vesicles (Linke, 1933; Fretter and Graham, 1962; Bedford, 1965), in the seminal receptacle (Bedford, 1965) and in the testis (Linke, 1933).

Phagocytosis of sperm in *L.sitkana* and *L.scutulata* has been studied with light and electron microscopy using tissue of the seminal vesicles. The cells of the seminal vesicle (S-V) wall undergo a cycle, as proposed by de Jong Brink (1969), in which a resting cell becomes phagocytic and later destructive. The cycle occurs throughout the year but is precipitated by certain conditions such as; starvation, parasitisation, and the termination of the breeding season. It has been reported that gamma radiation also induces the phagocytic process (Joosse et al., 1968).

Ripe or sperm-packed seminal vesicles appear pearly white. During sperm resorption, the duct is brownish, though

patches of white sperm often can still be seen.

Observations

The cells of the S-V wall produce macro-apocrine secretions, which are shed into the lumen. These will be termed 'cell buds'. At the light microscope level phagocytosis is evident both in the lumen, where the cell buds can be seen containing coiled sperm, and in the cells of the cell wall, where one sees individual sperm coiled up and enclosed in vacuoles (PLATE 34A). The membranes of the S-V cells and cell buds are very labile and can expand readily to envelop sperm, or nurse cells, adhering to them. The sperm are resorbed either in their filamentous form , or in a highly coiled 'sperm ball'. This process is observed only at higher magnifications and greater resolution using transmission electron microscopy.

The cell cycle is continuous but it is possible to distinguish between resting cells, and phagocytic or destructive cells on the following morphological basis:

Resting Cell

S-V cells in the resting condition are of the cuboidal epithelial type with; a large basal nucleus, many small mitochondria, a few lysosomes, granular endoplasmic reticulum (much of which is in the form of an onion body - PLATE 28A, C), free ribosomes, one or more Golgi bodies

(and secretory vesicles produced by them), and a uniform microvillar apex lacking cilia. Adjacent cells maintain contact via desmosome-like junctions (PLATE 31E).

Phagocytic or Destructive Cell

S-V cells in the active condition are usually of the columnar-epithelial type. The nucleus remains basally located. There is a marked increase in the number of mitochondria, secretory vesicles, lysosomes, granular endoplasmic reticulum, and free ribosomes. Multivesicular bodies, large and small vacuoles containing degenerating sperm balls (or filaments), and myeloid figures, are also evident at this time. The apical surface is dramatically changed: Large buds are formed and sloughed off into the lumen; sperm balls are engulfed by the overgrowth of surrounding cell membranes; and microvilli are distorted and often filled with vacuoles and microvesicles.

The mitochondria are usually aggregated apically and basally. Basal mitochondria often appear to be contained in invaginations of the basement membrane, which may actually represent lateral extensions of an adjacent cell. Lateral extensions of other portions of cells in the active phase are often evident in sections. The above events are depicted in PLATE 32 .

Cell Buds

Cell buds contain lysosomes, multivesicular bodies, numerous microvesicles and secretion granules, large and small vacuoles, ribosomes and mitochondria. They do not have nuclei. PLATE 32 shows a cell bud in the process of formation.

The Phagocytic Process

It is accepted practice, when identifying the functional activities of lysosomes and their derivatives, to incorporate a variety of cytochemical, histochemical and tracer techniques in addition to morphological data (Smith and Farquar, 1966; Dingle, 1972).

In this study it has been possible to deduce the cellular processes involved, solely on the basis of morphological data, because the element being digested is the sperm whose ultrastructure has been examined in detail in the present study. Even in a late stage of digestion it is possible to identify fragments of sperm in a cell body and so define it with reference to previously established cycles of intracellular digestion (Maunsbach, 1969; Novikoff, 1969; de Duve and Wattiaux, 1966; Smith and Farquar, 1966). This is not possible in many other systems of this type. As de Duve said: "It appears that lysosomes are unique among cytoplasmic organelles in their ability to accumulate exogenous material. In fact the involvement of

certain cell organelles in such an aggregation of exogenous material can be used as a means for the identification of lysosomes".

For the purpose of describing the process of intracellular digestion of sperm in Littorina, the following definitions have been made:

1. Cell Bud - a membrane-bound vesicle secreted by S-V cells into the lumen, having the capacity for phagocytising and digesting sperm (PLATE 28B, D). A cell bud itself, can be phagocytised by an S-V cell (PLATE 28E).

2. Heterophagic Vacuole - a membrane-bound vesicle in the cytoplasm of an S-V cell (or cell bud), containing undigested sperm elements (PLATE 28B, D; 29A).

3. Primary Lysosome - a membrane-bound vesicle that apparently has not been previously involved in a digestive event. Usually secreted by the Golgi body (PLATE 29C, D). May be incorporated in a multi-vesicular body or other lysosomal derivative.

4. Multi-Vesicular Body (M.V.B.) - a membrane-bound vesicle containing several smaller membranous vesicles of the primary lysosome type. It can digest sperm (PLATE 29E), or become

incorporated into other lysosome- like bodies, heterophagic vacuoles, or cell buds, for this purpose (PLATE 30C).

5. Myeloid Figure - a series of membranous elements, usually densely packed, and often forming whorls. It represents a terminal product in the digestion of sperm (PLATE 30A, C, D).

6. Secondary Lysosome - a membrane-bound vesicle containing sperm elements in the process of digestion as shown by structural changes but not usually containing myeloid figures (PLATE 30C).

7. Telolysosome - a secondary lysosome in a late stage of digestion, usually with the presence of myeloid figures (PLATE 30E, F).

8. Residual Body - a membrane-bound vesicle with dense contents representing the last stage of the intracellular digestive system. Contains terminal products of digestion such as, myeloid figures, dense granules, lipoid bodies and vacuoles (PLATE 30 C, E; 31D).

9. Lipoid Body - a lightly staining, ovoid body that resembles other elements previously described as 'lipid droplets', or 'lipid bodies' (Smith and Farquar, 1966; Dingle, 1969; 1972). Can occur anywhere in the cell but more often

are basally located and are sometimes found incorporated in lysosomal derivatives, such as residual bodies (cf. Smith and Farquar, 1966). A lipoid body is thought to represent a terminal product of the digestion process and is probably composed of lipid and poly- saccharide (Tessenow, 1969).

Phagocytosis (endocytosis)

Sperm may be resorbed in two different ways:

1. The labile membrane of a cell bud in the lumen, actively overgrows and engulfs an adhering sperm filament, or spermatozeugma { sperm clump with nurse cell } (PLATE 28B, D).

2. An S-V cell membrane overgrows and engulfs an adhering sperm filament, spermatozeugma, or cell bud containing resorbed sperm (PLATES 28E; 29B).

In each case an heterophagic vacuole is formed. Subsequently, this is digested intracellularly by fusion with primary lysosomes, a multivesicular body, or another lysosomal derivative, to form a secondary lysosome. Destruction of sperm in a cell bud can begin before it is resorbed by an S-V cell (PLATE 28B, D, E).

Secondary lysosomes gradually pass to the telolysosome stage and subsequently become residual bodies, as the sperm are broken down into the terminal digestion products.

Myeloid figures gradually degenerate to form lipoid bodies and dense granules. In other systems, any lysosome-like derivative, such as a residual body or telolysosome, can fuse with any other member of the cycle and continue in the digestive process. It is likely that this also applies in *Littorina*. The largest residual bodies can occupy one-third of the contents of an S-V cell.

Indigestible material may pass out of the cell by exocytosis, which has been reported elsewhere (de Duve, 1969; Maunsbach, 1969), but has not been observed in this study.

The main events of sperm resorption and digestion are summarised in PLATE 32, and a hypothetical cycle of intracellular digestion of sperm is illustrated in PLATE 33.

Discussion

Very little research has been done on sperm phagocytosis in molluscs, in spite of its recognition in earlier studies (Linke, 1933; Fretter, 1941). Most recent research relating to molluscs has been conducted at the light microscope level and then only superficially (Bedford, 1965; Ponder, 1972). A few investigations have been made with the electron microscope (Breucker, 1964; de Jong Brink, 1969) but they did not attempt to describe the cellular processes involved. One of the main reasons that this topic has been left alone, is because of the difficulty of

achieving adequate fixation of highly active cells that are already involved in processes of degradation. Improved fixation techniques have alleviated this problem. A second difficulty is that residual bodies are often extremely dense and cause streaking across sections.

The process of intracellular digestion of sperm involving the lysosome system of the cell, bears close similarity to heterophagic and autophagic digestion; in mammalian tissues (de Duve and Wattiaux, 1966; Smith and Farquar, 1966; Holtzman and Novikoff, 1965; Mahler and Cordes, 1966; Holtzman, 1969); in invertebrates such as the fresh water sponge, during vitelline platelet digestion in gemmules (Tessenow, 1969); and in insects, during metamorphosis (Lockshin, 1969).

The history of the lysosome concept was reviewed by de Duve (1969) , where he noted that Metchnikoff discovered intracellular digestion in the planarian, Geodesmus bilineatus in 1865. Formulation of the basic concepts of the phagocytic process were made by Metchnikoff in the period 1865-1901 and by Volkonsky, between 1929 and 1934. Lysosomes were first recognised as a separate category of cell organelles by de Duve et al. (1955). Since then the literature has been inundated with various articles on the subject (for reviews, see Dingle, 1969; 1972; 1973).

The phagocytic process in Littorina differs from that of the other examples, in the mechanism of secretion of cell

buds and in the process of endocytosis. The formation and release of cell buds that have the capacity to phagocytise and digest waste elements, has not been reported previously. Macroapocrine secretions of this nature usually do not contain cell organelles such as lysosomes, or mitochondria (Kurosumi,1961).

The endocytosis of sperm balls and cell buds by S-V cells, closely resembles the uptake of particles by amoebocytes found in the interstitial fluid (PLATE 31B). This process also occurs in the amoebocytes and leucocytes of mammalian tissues. However, the endocytosis of sperm filaments is different, as this involves extension of the cell membrane on one side, wrapping up the sperm filament in a series of whorls and gradually enclosing the sperm inside the S-V cell by fusion of adjacent internal membranes (PLATE 31A, C). To my knowledge this method of endocytosis has not been previously described in this context, although a similar process occurs in the formation of the myelin sheath of nerve axons.

Myeloid figures (membranous whorls) have been noted in a wide variety of cells (see review by Ruby and Webster, 1972). Besides their presence in normal processes of cell degeneration (Fawcett and Ito, 1958; Ito, 1962; Swift and Hruban, 1964; Holtzman and Novikoff, 1965; Sun, 1966; de Duve, 1969) myeloid figures have been attributed to a variety of other factors such as; cell stress, cell

pathology, changes in protein nutrition, production of cell organelles (Golgi body) and poor fixation technique (see review by Ruby and Webster, 1972, for references). They have also been created artificially from phospholipids and pure egg lecithin in an aqueous phase, but in these cases the myelinics are only preserved when glutaraldehyde fixation precedes osmium treatment (Sun, 1966). Crurgy (1968) believes that myeloid figures are artifacts of glutaraldehyde fixation but this has been disputed by Aaronson et al. (1971), who demonstrated clearly that multi-membranous systems are seen in tissues, when either osmium or glutaraldehyde is used as the primary fixative.

The myeloid figures in the present study are not considered to be artifacts of fixation, because they were also found in fresh frozen tissues employing freeze-etch techniques (PLATE 30B). Additionally, previous studies on cell degeneration (mentioned above), and on other phagocytic cells (Gezelius, 1959; Mercer and Shaffer, 1960; Vickerman, 1962; Swift and Hruban, 1964; Smith and Farquar, 1966), have repeatedly shown their presence in spite of the use of a variety of fixation techniques.

The membranes of the lysosome system must be different from those of the Golgi , endoplasmic reticulum and nucleus, in order to prevent indiscriminate fusion and hence cell destruction (Dingle, 1969; 1973). This brings back the idea of the 'vacuome' (Dangeard, 1956; Parat, 1928), which was

reappraised by de Duve and Wattiaux (1966) and de Duve (1969). These authors introduced the idea of the endoplasmic and exoplasmic spaces. They envisage a one-way lock between endo- and exoplasm caused by the changeover from one membrane type to the other, such as in the formation of a secretion granule from the Golgi body. In support of this concept, Yamamoto (1963) had previously shown that the membranes of some organelles are thinner than those of others. These two groups are now considered discrete and have been assigned to the endo- and exoplasmic spaces as follows:

1. Endoplasmic; mitochondria, granular and agranular endoplasmic reticulum, Golgi lamellae.

2. Exoplasmic; plasma membrane, lysosomal membranes, secretion granules, Golgi vesicles.

Grove et al. (1968), working with fungal hyphae, found that; "a major function of the Golgi apparatus is to elaborate secretory vesicles whose limiting membranes can fuse with plasma membranes". Membranes of secondary lysosomes have been shown to be partly plasma membrane in origin (Thines-Sempoux, 1967). De Duve (1969) emphasises the idea that many cell processes in the exoplasmic space occur independently and this would only be possible if some change occurred in the interconversion of the organelle membranes during membrane fusion.

In Littorina the Golgi body, agranular and granular endoplasmic reticulum, are often very closely associated, to the point where it is sometimes difficult to distinguish between them - such as with the onion body in PLATE 28A, C . It is very possible that these elements are interconnected and form a contiguous system like the 'vacuome' concept. Onion bodies, similar to the ones observed in this study, have been seldom found before. The only case that is close enough to be worth mentioning, is found in sperm of the arthropod, Bacillus rossius (Baccetti et al., 1973). These authors describe it as; "an organelle system consisting of laminae radiating from common centres" and interpret it as being; "a mechanism of cytoplasm degeneration", since degenerating mitochondria can be seen embedded in it. They discovered the membranous body in a degenerating portion of spermatid cytoplasm.

The onicn body of Littorina S-V cells is often closely associated with numerous granular and clear vesicles. It seems likely that, since it appears more active during the phagocytic or destructive stage, that it is involved in the processing of substances, possibly lysosomal, for the digestion of resorbed sperm.

THE FEMALE REPRODUCTIVE SYSTEM

PART 1: L.SITKANA

General Description

The ovary is a diffusely-branching, orange mass embedded in the digestive gland. It varies in extent throughout the year occupying up to three-quarters of the the digestive gland complex during the breeding season, at which time it is filled with ripe oocytes.

The ovarian tubules drain into the oviduct which passes close to the surface, as a thin-walled tube, just below the pericardial cavity. A ciliated gonopericardial duct connects the oviduct with the pericardial cavity. The pericardial cavity is connected with the kidney and mantle cavity via the renopericardial canal and kidney aperture. Just anterior to the gonopericardial duct and adjacent to the albumen gland, the seminal receptacle duct connects the oviduct with the receptaculum seminis, which is a 1mm long, blind-ended sac, functioning as a sperm storage organ.

The oviduct follows a complex pathway through the pallial region in which are located three glands: The albumen gland, capsule gland and jelly gland (PLATE 35A.) The posterior portion of the albumen gland produces a thick granular secretion around the egg, which is then enclosed in

a membrane secreted by the anterior portion. Individual eggs are encased in a transparent, rigid capsule secreted by the capsule gland. Finally 100-300 eggs are embedded in a gelatinous secretion, produced by the jelly gland, which forms the egg mass. The egg mass accumulates in the vestibule and is gradually extruded out the gonopore by muscular action. The ovipositor, an upraised area of glandular tissue located on the foot below the right cephalic tentacle, secretes a sticky, mucoid substance over the egg mass, which probably attaches it to the substrate.

Close to the gonopore a large blind-ended sac, called the bursa copulatrix (which may be referred to as the 'bursa' in future), opens into the oviduct. From where the bursa meets the oviduct a channel runs directly to the opening of the receptaculum seminis into the albumen gland. This channel, which is actually part of the oviduct, provides a direct route for sperm between the bursa and the receptaculum seminis, where they are stored.

The functional morphology of the female genital tract will be described by tracing the path of male and female gametes into and out of the system, beginning with the deposition of a spermatophore in the bursa by the male.

A dissected female has been drawn to show the relative positions of reproductive glands and organs (PLATE 4B).

Bursa Copulatrix

The bursa is a sac-like organ measuring about 3mm in length and lying adjacent to the mantle cavity below the rectum. It has one entrance which opens inside the oviduct just behind the gonopore.

The bursa wall is composed of epithelial gland cells which vary from cuboidal to columnar in shape. The main body of the gland is usually composed of unciliated gland cells which have a well-developed microvillus border. However, in the anterior portion of the bursa, closer to its connection with the oviduct, the gland cells bear cilia (PLATE 36A). Some bursa cells contain a red pigment enclosed in a vacuole, which probably represents lipofuscin, the characteristic degeneration product of an aging cell (Bloom and Fawcett,1968). The pigment has an amorphous appearance when viewed electron microscopically (PLATE 36B) but in thick sections it has a reddish colour. Other cells contain a mucus secretion which, in thin section, appears as moderately dense granules enclosed in a vacuole (PLATE 36B).

All the bursa cells undergo an apocrine secretion process in which cytoplasmic buds form at the cell apex and are released into the lumen (PLATES 36A,C and 37A, B). This process is similar to that in the human prostate gland (Bloom and Fawcett, 1968) and human terminal bronchioles (Mathews and Martin, 1971) and is also found in the resorbing cells of the seminal vesicles of *Biomphalaria*

glabrata (de Jong Brink, 1969) and Littorina (see previous section). Cytoplasmic buds have a pale translucent appearance and contain fine secretion granules but lack cell organelles. Sperm heads often become closely associated with the cytoplasmic buds and also with individual microvilli but they do not penetrate the cells or become invaginated by them. The bursa cell has a large median nucleus with prominent nucleoli; Golgi bodies; lipid droplets; and many apical mitochondria. The lower half of the cell tapers to a fine process before butting onto the basal lamina. This inverted, pyramidal shape enables the production of folds in the bursa wall which provide a greater surface area for secretion. Below the basal lamina is a muscle layer which forms a continuous sheet surrounding the bursa .

When a spermatophore is deposited in the bursa, the seminal fluid and prostatic secretions are rapidly dispersed and the nurse cell membranes are destroyed. The liberated sperm, which have been activated during the transition from male to female snail, orient themselves with the wall of the bursa; heads toward the cells, tails beating sychronously in the lumen (PLATE 34B). Some sperm are expelled from the bursa by muscular contractions of its wall into the ventral channel of the oviduct and from there swim to the receptaculum seminis (a sphincter muscle encircling the adjacent renal oviduct prevents premature contact between sperm and eggs). Sperm remaining in the bursa for longer periods (more than a week) are digested extracellularly.

Receptaculum Seminis

When the sperm reach the receptaculum seminis they become embedded, head first, in invaginations of the epithelial cells (PLATE 38 A-D) their tails beating synchronously in the lumen. Often the head becomes very deeply invaginated and penetrates as far as the nucleus, which is located below the centre of the cell (PLATE 38B, D).

The cells of the wall of the receptaculum seminis are the columnar epithelial type with a dense border of microvilli at the apex. The cells have numerous mitochondria in the apical third together with occasional lysosomes. The prominent Golgi body, usually with several Golgi vesicles, is situated above the nucleus.

Mixed endoplasmic reticulum is scattered throughout the cell, though one tends to find more between nucleus and mitochondria. The basal lamina is irregularly folded along its length.

Bounding this layer of epithelial cells is a thick layer of longitudinal and circular muscles. This muscle sheath surrounds the entire organ and may be responsible for the rapid expulsion of sperm.

The general appearance of the cells of the receptaculum seminis are illustrated in PLATE 38C .

Sperm may be stored in this receptacle for at least one month. During this time they are perpetually beating. Sperm digestion by phagocytosis has not been observed in the receptaculum seminis. However, in parasitised animals the cells of the seminal receptacle duct develop a variety of different secretions, which appear to digest sperm extracellularly.

When the sphincter muscle surrounding the exit from the renal oviduct relaxes, the eggs are expelled in a continuous stream past the opening of the seminal receptacle duct, into the albumen gland. It seems likely that at this moment the sperm are expelled by contraction of the muscular wall of the receptaculum seminis in order to fertilise the eggs. The most probable site of fertilisation is indicated in PLATE 35A, B.

Detailed light microscope observations on the morphology of female _Littorina_ have been made previously by Linke (1933) and Fretter and Graham (1962). The morphology of _L.sitkana_ is close to that of _L.obtusata_, whereas the morphology of _L.scutulata_ is very similar to that of _L.littorea_. However, the oviduct system in _L.sitkana_ and _L.scutulata_ is quite different from the interpretation given by these authors (cf. PLATE 35).

The fine structure of the different gland cells of the pallial oviduct has not been studied in detail, since the

intermingling of gland cells has made it very difficult to locate a specific region. However, where sufficient evidence has been accumulated a description will be included. Additionally, a general account of morphology will be given, noting where differences arise between the present study and previous accounts of prosobranch morphology.

Pallial Oviduct

Albumen Gland

The eggs are embedded in a thick granular albumen secretion which provides nourishment for the early veligers in the capsule and enables them to develop to the juvenile stage before hatching.

The albumen secretion is produced in glandular epithelial cells lacking cilia. Each secretion droplet is composed of fine granules. Similar granules are found in the albumen gland of *Helix pomatia* (Nieland and Goudsmit, 1969). The size of the secretion granules varies, depending on the reproductive state of the animal. During the breeding season the cells are roughly pyramidal in shape and the secretion granules almost completely exclude the remaining cytoplasm. The nucleus occupies a basal position. At other times, such as at the end of the breeding season, or in a lightly parasitised individual, the cells become more cylindrical in shape. In these situations, the secretions vary greatly in

size and only occupy a portion of the cytoplasm and the nucleus becomes more centrally located (PLATE 39A).

The gland cells have large elongate mitochondria, Golgi bodies, and a rich endoplasmic reticulum which is often arranged in complex whorls surrounding secretion granules. The apical surface is covered with a sparse layer of microvilli. The lower portion of the cell tapers to a fine process which is often thrown into several folds before reaching the basal lamina.

The gland cells are supported by ciliated, columnar epithelial cells which extend from the basal lamina to the lumen, often as very fine processes, separating adjacent gland cells. The apical surface bears numerous cilia separated by microvilli. The nucleus is located below an apical aggregation of mitochondria.

Ciliated cells occupy most of the apical surface and gland cells empty their contents in small depressions between them. These observations are similar to those made by Nieland and Goudsmit (1969) . The section of oviduct in the albumen gland, when expanded, forms a spiral (PLATE 35A). At the base of the spiral is located the region of the albumen gland that produces the egg covering in L.sitkana, which is a thin membrane enclosing the albumen and egg. However, attempts to show any differences in fine structure between cells of this region and those of the albumen gland proper have been unsuccessful. Owing to the different

staining properties of the two regions, one might expect some difference in gland cell morphology (see summary of staining properties of glands at end of section). However these stains are inadequate for obtaining any detailed histochemical information.

Capsule Gland

The oviduct follows, approximately, a U-shaped course through the capsule gland as depicted in PLATE 35A. The gland is composed of three regions: an inner layer, a median layer, and an outer layer (PLATE 42A). Inner and outer layers are identical, the median layer is different. These regions are so arranged that an egg passing through them in the oviduct, recieves two different secretions, each one composing half of the capsule. Mixing of secretions occurs where the walls of the oviduct press the two halves of the capsule together. The two materials composing the egg capsule are rendered visible with Masson's Trichrome stain (PLATE 42B). The glandular regions are not completely distinct as sometimes one finds an intermingling of cells.

Capsule gland secretions tend to be drawn out into long strands by ciliary action, creating a meshwork of fibres interspersed with secretion droplets (PLATE 40A). The characteristic shape of the capsule is formed in the capsule gland and not in the ovipositor, or vestibule.

Jelly Gland

The completed capsules pass on into the jelly gland where they are embedded, en masse, in a fibrous jelly secretion. The jelly secretion is produced by extremely elongate cells which may reach at least 0.5mm in length (PLATE 39B). The secretions are produced in the form of granules arranged in single file along the entire length of the slender cell body. The nucleus is basally located and, as in other gland cells, whorls of granular endoplasmic reticulum surround secretion granules. At the cell apex the secretions are released in small depressions between large, ciliated, epithelial, supporting cells. Both types of cell have microvilli on the apical surface but only the supporting cells bear cilia. In ciliated cells the large nucleus is always apically located and has an irregular, lobed appearance. The secretion granules fuse in the lumen of the gland and form long fibrous strands (PLATE 42C). The entire jelly gland and its duct form a series of distinct ridges which provide a larger surface area for secretion (PLATE 35A).

The egg mass collects in the vestibule and is gradually extruded out the gonopore by muscular action, past the ovipositor and so onto the substrate. A diagram of a single completed egg capsule is shown in PLATE 2E.

PART 2: L.SCUTULATA

General Description

The female system of *L.scutulata* is similar to that of *L.sitkana* with respect to: The structure and point of insertion of the bursa; the seminal receptacle, and the renal oviduct; and the presence of a capsule gland. However it differs because of: the shape of the pallial oviduct; the absence of albumen and jelly glands; the presence of a covering gland; and the appearance of the ovarian yolk. In *L.scutulata* no trace of albumen has been observed inside egg capsules that are in the process of formation within the pallial oviduct (PLATE 42D). It appears then, that the covering gland completely replaces the albumen gland in this species. A dissected female, showing the main features of the genital system, is illustrated in PLATE 5B.

Pallial Oviduct

The oviduct follows a complex spiral path through the covering gland. The seminal receptacle duct and renal oviduct are inserted near the top of this spiral . The most likely site for egg fertilisation is indicated in PLATE 35B.

The eggs that have entered the covering gland are surrounded by a relatively thick egg covering, compared to that enclosing the albumen and egg of *L.sitkana*.

The cells of the covering gland are similar to those of the capsule gland (PLATE 40B) in this species. Gland cells containing secretions with a fine punctate appearance (PLATE 41B) are supported by ciliated epithelial cells. The latter have apical nuclei and a densely ciliated surface interspersed with microvilli. Their cell processes extend to the basal lamina. The gland cells have nuclei near the middle or base of the cell. As in other gland cells the secretions often appear within whorls of granular endoplasmic reticulum (PLATE 41B). In addition to these formations, endoplasmic reticulum is scattered throughout the cytoplasm. The cell apex terminates in a small depression between ciliated cells and in this region one finds microvilli (PLATE 41A). Adjacent cells are in contact via desmosome-like junctions . Mitochondria are aggregated at the cell apex together with scattered dense bodies, which resemble the residual bodies of the lysosome system. In each cell one finds a strange vesicular structure, along one side of which are a row of small tubules (PLATE 41A). The tubules appear circular when cut in the longitudinal plane of the vesicle but have a tubular appearance in sections perpendicular to this. The membranes of the vesicular structure measure only 55A across, compared to 75A for the adjacent plasma membrane in PLATE 41C. The function of this vesicle and the adjacent microtubules is unknown.

Capsule Gland

In the capsule gland 1-5 eggs are first attached to each other by strands of secretion. Subsequently the capsule is deposited around the eggs with some of the strands adhering to the inside surface, such that, in the completed capsule, the eggs are separated by fibrous interconnections and also are individually attached, in several places, to the capsule wall (PLATES 34C, 43).

In *L. scutulata* egg capsules appear to be moulded into their characteristic helmet shape by the muscular walls of the vestibule and not in the capsule gland, as in *L.sitkana*. The ovipositor seems to be absent in *L. scutulata*. Observations on egg-laying snails indicate that the capsules pass directly out of the mantle cavity into the sea water without receiving secretions or final moulding by any part of the foot (ovipositor). The two species may be different in this respect.

The staining properties of the glands of the pallial oviduct in female *L.sitkana* and *L.scutulata* are summarised below and in Table 1:

1. The secretions composing the egg covering and that portion of the egg capsule that derives from the medial lobe of the capsule gland in *L.sitkana*, stain similarly to the secretions forming the egg covering and egg capsule of *L.scutulata*.

2. The secretions forming the albumen, surrounding the egg of L.sitkana, are stained blue by Masson's Trichrome and green-blue by the AB/AY technique. No other secretion type stains in this way.

3. The secretions composing the jelly gland of L.sitkana are not stained by Masson's Trichrome but stain blue-green by the AB/AY technique. No other secretion type stains in this way.

4. The secretions forming the portion of the egg capsule that derives from the lateral lobes of the capsule gland in L.sitkana stain red with Masson's Trichrome and yellow with the AB/AY technique.

5. In conclusion, it appears that one component of the egg capsule, the albumen secretion, and the jelly secretion are present only in L.sitkana , whereas other secretions are similar in both species.

PART 3: DISCUSSION

Bursa Copulatrix and Receptaculum Seminis

There has been much confusion in the literature as to the location and function of these two organs. The problem has been reviewed in opisthobranchs by Thompson and Bebbington (1969), Beeman (1971) and Schmekel (1971); in pulmonates by Duncan (1958); in stenoglossans by Fretter

(1941), Fretter and Graham (1962), and Ponder (1972); and in mesogastropods by Ghiselin and Wilson (1966), and Johannson (1953, 1957). In Littorina the bursa copulatrix is the sperm receiving organ situated close to the gonopore, whereas the receptaculum seminis is the sperm storage organ situated at the posterior (or proximal), end of the oviduct.

Bursa Copulatrix

In general the functions of the bursa appear to be: 1. Spermatophore reception; 2. Temporary storage of sperm; 3. Sperm liberation; 4. Resorption of nutrients derived from the breakdown of nurse cells and prostatic secretion; 5. Extracellular digestion of left-over sperm.

The bursa secretions may also affect the activity or capacity of sperm. In Littorina, contact with sea water activates sperm which have been removed from the testis or seminal vesicles. However in many gastropods the sperm are non-motile when delivered to the bursa, but subsequently become activated by the secretions of the bursa (Thompson and Bebbington, 1969; Beeman, 1971). In Littorina the sperm are active before they reach the bursa. It is tempting to assume that since contact with sea water activates them, this is the sole activating agent. However, contact with the prostatic secretion may also initiate motility. Interestingly, Omura's (1950) studies showed that in the moth, Bombyx mori, spermatozoa only became fully fertile after contact with secretions of the lower portion of the

male tract. The penial glands of L.sitkana are unlikely to be involved in this, as they are not placed in a suitable position to meet the spermatophore and also this would not apply to L.scutulata, in which the glands are absent.

Another possibility is that the sperm acquire their full fertilising capacity within the female tract. Duncan (1958) found the enzyme acid phosphatase localised around the free borders of the glandular cells of the bursa of a fresh water snail, Physa fontinalis. He proposed that the enzyme served a nutritive function and that "the (bursa) secretions taken together may produce a stimulating effect on sperm motility or power of fertilisation". He also noted that acid phosphatase is a constituent of the mammalian prostatic secretion, possibly serving in the liberation of fructose necessary for the nourishment of the spermatozoa (Pearse, 1953). This is an interesting point, because the cells of the human prostate produce macro-apocrine secretions in a very similar way to those of the bursa cells of Littorina.

The idea that the breakdown products of the prostatic fluid and apyrene sperm provide nutrients for the eupyrene sperm was proposed by Dembski (1968), working with Viviparus contectus. In mammals, under normal conditions, a wide variety of glycolysable substances are present in the semen, which provide the main source of energy for spermatozoan metabolism (Mann, 1964). However it must be emphasised that

acid phosphatase is a digesting enzyme and a characteristic constituent of lysosomes. In fact it is widely used for positive identification of lysosomes (Smith and Farquar, 1966). Lysosomes are usually involved in the breakdown of various proteins, either foreign or cytoplasmic, as occurs in the seminal vesicle cells of Littorina during sperm resorption. Thus, the apocrine bursa secretion may have more than one function: on a short term basis it may destroy the nurse cell membranes and disperse the prostatic fluid, possibly making nutrients available to the sperm and bursa cells; on a long term basis it may digest old sperm extracellularly - the nutrients formed from this process are probably resorbed, as occurs in Lymnaea stagnalis (Horstman, 1955). It is unlikely that the other bursa secretions have these properties , since one is a pigment, which is probably lipofuscin and represents the degeneration product of an ageing cell (Bloom and Fawcett, 1968), and the other is a mucus substance. Extracellular digestion of sperm in the bursa (or its homologue), in other molluscs, has been reported by several authors (Duncan, 1958; Fretter and Graham, 1962; de Jong Brink, 1969; Thompson and Bebbington, 1969; Beeman, 1970; Brandriff and Beeman, 1973).

Receptaculum seminis

Recent studies on spermatozoan metabolism have shown that complex enzyme systems are available in the mitochondrial and tail regions of prosobranch (Littorina

littorea) and pulmonate sperm (Helix, Helisoma - species not recorded) , for the utilisation of large glycogen reserves stored in these regions (Anderson and Personne, 1969b; Anderson and Personne, 1970a; Anderson, 1972). These authors believe that "endogenous glycogenous stores in these spermatozoa represent an important source of glycolysable material for the production of energy". They analysed the sperm of Helix, Helisoma and Otala (species not recorded), which have intracellular glycogen stores and found that they contained the enzymes necessary for the breakdown of glycogen - namely G.A.P.D. (glyceraldehyde-3-phosphate dehydrogenase) and L.D.H. (lactate dehydrogenase). They concluded: "These findings demonstrate, therefore, that the dehydrogenases that catalyse the oxidation-reduction reactions of the glycolytic pathway are localized mainly in the glycogen compartment, but are present in the matrix compartment as well. These findings further support the suggestion that the glycolytic enzymes are located in close proximity to the glycogen reserves within the mitochondrial derivatives of sperm of pulmonate gastropods".

In the seminal receptacle numerous, synchronously-beating sperm are stored tightly packed together. As was mentioned in a previous paper (Buckland-Nicks, 1973), the filiform nature of Littorina sperm and the fact that the mitochondrial region is in contact with the external environment via a series of pores, are adaptations suited to conditions of low oxygen tension (Anderson and Personne

1969b, Anderson and Personne, 1970a; Anderson, 1972).

In some gastropods, the seminal receptacle cells may provide nutrition since several workers have recorded the presence of secretory granules in the membranes surrounding the sperm. In opisthobranchs, two different situations are found. Schmekel studied 11 different species and concluded that the "heads of the allo-sperms are deeply embedded in the epithelial cells...... the outer membrane of the sperm and plasmalemma of the receptaculum cell lie close together. The close contact of the two membranes and the reaction of the epithelial cells seems to indicate a very tightly knit relationship between the epithelium and the sperm. Quite likely the sperm receives nourishment from the receptaculum cell". Thompson (1966) and Thompson and Bebbington (1969) studied the same association in Archidoris pseudoargus, Aplysia depilans, A. fasciata and A. punctata, which have a more filiform head shape, and concluded that each sperm head becomes entwined with a microvillus but does not penetrate the cell within an invaginated cell membrane. Thompson believed that the microvilli serve a nutritive function.

In Littorina the sperm are deeply embedded in invaginations of the receptaculum cells. However, secretory material has been observed near the sperm, which may indicate that they have alternative means of obtaining nutrients, such as that suggested through the activity of L.D.H. under low oxygen tensions.

The Pallial Oviduct

The pattern of alternating gland cells and ciliated cells is a common feature of the pallial oviducts of molluscs. According to Ranvier's (1887) classification, all the gland cells of the pallial oviduct of Littorina are merocrine. Those of the albumen, covering, capsule and jelly glands are 'eccrine', producing secretion granules within the cytoplasm and releasing them apically as individual packets without other cytoplasmic loss, or cell destruction. The gland cells of the bursa are mixed (apocrine and eccrine) since they produce both macro-apocrine secretions (that collect at the free borders of the cells), and also a mucus secretion of the eccrine type. For this reason bursa cells resemble gland cells of the mammalian apocrine and eccrine sweat glands, thyroid gland, and mammary gland.

Secretion Formation

The Golgi body has long been considered the most significant organelle in the secretion process (Bowen, 1924; Hirsch, 1939). In his review of the secretion mechanism, Kurosumi (1961) pointed out that the ergastoplasm (granular endoplasmic reticulum) has been shown, by many authors, to be important in the synthesis of secretions. Some argued that the ergastoplasm is the chief site of production of zymogen granules in the exocrine pancreas (Weiss, 1953; Suzuki, 1958; Jamieson and Palade, 1957a, b). However

evidence was also produced in favour of the Golgi body as being the key site for synthesis. Hendler et al. (1957) found that in the albumen-secreting gland of the Hen oviduct, the well-developed ergastoplasm is often observed containing secretion material, while the Golgi body remained clear and empty. Kurosumi believes that the secretion forms in the ergastoplasm and is transferred to the Golgi, where it is concentrated into vesicles. This supports some early light microscopists' idea of the 'vacuome' (Dangeard, 1956; Parat, 1928). For further discussion see section on 'Sperm Resorption' .

In the gland cells of the pallial oviduct of Littorina, one often sees extensive whorls of granular endoplasmic reticulum containing secretions similar to those in the mature granules within the cytoplasm. Similar observations have been made in a variety of nudibranchs (Schmekel, 1971). Although Golgi bodies are present, secretion granules have not been frequently observed in association with them. This might indicate that the cells pass through different phases of a cycle, synchronously, such that, at any one time, they will be either synthesising, concentrating, or releasing secretion granules. At any rate it appears that the granular endoplasmic reticulum plays an important role in sythesising secretions in the albumen and jelly glands of L.sitkana and in the covering and capsule glands of L.scutulata. Possibly these secretions are later condensed and packaged by the Golgi body as suggested by Kurosumi (1961) and Fawcett

(1966).

<u>Albumen Gland</u>

The absence of albumen from the egg capsules of *L.scutulata* is in keeping with the short planktonic life of the larvae. Food for the embryo is restricted to the yolk supply in the egg which is sufficient for development to the veliger stage. Conversely, the large quantity of albumen in *L.sitkana* egg capsules enables the embryo to develop directly to the juvenile stage, within the protective egg mass, before hatching out.

The albumen secretion has been studied histochemically in a variety of molluscs (Holm, 1946; Meenakshi, 1968; 1969; Duncan, 1958; de Jong Brink, 1969; Nieland and Goudsmit, 1969). These authors, and many others, found galactogen and proteins to be the main constituents of the albumen gland. Goudsmit (1965; 1966) found galactogen-synthesising enzymes in the albumen gland. He believed that this might account for the presence of proteins.

In *L.sitkana* the albumen gland and its secretion stained blue-green with AB/AY, which indicates the presence of an acid mucopolysaccharide. However de Jong Brink (1969) obtained a negative result using the same technique on *Australorbis glabratus*. *L.sitkana* may be unusual in this respect.

Capsule Gland

The incorporation of a fibrous matrix of capsular material between the eggs and the internal surface of the capsule in L.scutulata may be important for; 1. Maintaining the spatial relationships of the eggs within the capsule; 2. Providing additional strength and rigidity to the capsule; and 3. Enhancing the formation of the characteristic 'helmet' shape.

The heterogeneous nature of the capsule wall of L.sitkana may mean that some parts of the wall become weakened more rapidly than other parts. A progressive weakening of the capsule has been noted in both species although it was not possible to detect differences in strength between the two halves. This has not been included previously, since no precise techniques were designed to test it. However, when piercing the capsules with glass needles to ensure a good penetration of araldite embedding medium during fixation procedures, it was much easier to penetrate capsules containing veligers, than it was to penetrate those containing embryos in early cleavage stages. It is possible that the blue-staining, mucoid component of the egg capsule, which is present in both species, softens more rapidly than the red-staining, proteinaceous component, observed only in the capsule of L.sitkana. This, together with the fact that the capsule of L.scutulata is much thinner (on top and bottom) than that of L.sitkana, would

certainly be adaptive for the short planktonic existence of the former.

Jelly Gland

The secretion of the jelly gland may be important for a variety of reasons: 1. It supports the egg mass, and separates developing embryos such that conditions of overcrowding (the build up of metabolites), do not occur; 2. It protects the egg capsules, since it is both tough and resilient. 3. Owing to its fibrous nature, it is permeable to water and salts and so enables the correct osmotic balance to be maintained; 4. It is hydrophilic, thus limiting water loss under dessicating conditions; 5. It acts as a food source to juveniles when they hatch out; 6. It acts as a substratum for numerous microscopic organisms which may be involved in symbiotic relationships with the developing embryos (see APPENDIX 1).

Vestibule

The existence of a vestibule in Littorina is well established (Linke, 1933; Fretter, 1941). Its function is still obscure, although in some groups it acts as a site for sperm reception.

In L.scutulata it plays an important part in moulding the egg capsule into its final shape. It also produces a mucoid secretion which may aid in hardening the capsule. The presence of this sticky substance will often cause the

capsules to adhere to nearby rocks, or floating debris. This could have some advantage, by providing protection and a more stable substratum from which to hatch out.

In stenoglossans, the egg capsule is shaped and hardened by the muscular action and secretions of the ovipositor and 'ventral pedal glands' (Ankel, 1929; Fretter, 1941; Ponder, 1972). It should be noted that Linke (1933) proposed that the egg capsule of *Littorina littorea* is moulded into shape by an ovipositor and that its secretions caused hardening of the capsule. This is not true for either *L.sitkana*, or *L.scutulata* , since the egg capsules are fully formed when they leave the gonopore.

OVIPOSITION AND LARVAL DEVELOPMENT (with discussion of some factors affecting the breeding seasons).

INTRODUCTION

It is generally recognized that there are three main patterns of larval development in Littorinidae: (1) those with pelagic egg capsules and planktotrophic larvae; (2) those with benthic egg masses and lecithotrophic larvae; and (3) viviparous species. The first group can be further divided according to the shape of the egg capsules, e.g.; helmet, simple drum, drum and disc shape (Kojima 1960).

This chapter describes both field and laboratory observations on the oviposition and larval development of L.sitkana and L.scutulata, and includes a discussion of the effects of various environmental factors on the breeding seasons of both species.

MATERIALS AND METHODS

In the intertidal tide pools of False Bay, egg masses of L.sitkana are found attached beneath rocks and driftwood, or on macroscopic algae such as Ulva sp. and Fucus sp. About one week after laying they acquire a brownish appearance due to the layer of diatoms adhering to the jelly. In the laboratory L.sitkana deposit egg masses either on algae, on rocks, or on the sides of the tank in which they are kept. Egg masses were removed and cultured in small dishes at a temperature of 10-14°C on the water table. The sea water in the dishes was changed frequently and the development from fertilisation through hatching of the juveniles was examined and photographed under a Wild dissecting, or compound, microscope.

For the study of spawning in L. scutulata, snails were placed in covered Syracuse dishes, or finger bowls, with a small amount of filtered sea water and were kept either on water tables (10-14°C) or at room temperature (22°C). The dishes were examined daily and the number of egg capsules produced was recorded. After hatching, the veligers were cultured in small finger bowls which had been placed in sea water for at least a week so that a layer of diatoms and bacteria was well established on the glass wall. The larvae were fed either with a pure culture of Dunnaliella sp., or a mixture of diatoms and dinoflagellates collected from the tide pools at Friday Harbour (F-S Chia, 1970-1974).

OBSERVATIONS

PART 1. OVIPOSITION

L.sitkana and *L.scutulata* are both dioecious. During copulation the male positions itself on the shell of the female and the penis is inserted under the right side of the female mantle into the bursa copulatrix where the sperm and prostatic secretions are deposited (PLATE 7D). The spawning of both species usually occurs from 6 a.m. to 9 a.m. and from 6 p.m. to 9 p.m. in the laboratory during the spring and summer, which indicates that it may be under the influence of changing light intensity on a diurnal basis. The effect of light on spawning is found in many molluscs as well as other marine invertebrates (Barnes, 1963; Chia, 1971b; Kume and Dan, 1968; Segerstrale, 1970).

L.sitkana

Although egg masses may be found in the field all year round, maximum spawning is biannual; one peak occurs in April and another in October (PLATE 44).

Usually each female lays between 50 and 400 eggs, which are sequentially fertilised as they enter the albumen gland from the renal oviduct. The eggs are embedded in albumen and subsequently individually encapsulated by an inner, thin egg covering (chorion) and an outer, thick

capsular wall. The encapsulated eggs are enclosed in a thick, transparent, gelatinous material forming the bulk of the egg mass, which collects in the vestibule and passes out of the gonopore, along the right hand side of the head, where it is covered with a sticky secretion by the ovipositor, and deposited on the substrate.

The diameter of each egg is about 175um Granular albumen surrounds the egg and fills the rest of the capsule, the latter being transparent and rigid with a maximum diameter of 1 mm (PLATE 2E).

Individual egg masses vary in length from 5mm to 15mmbut often epidemic spawning by a number of females results in a large egg mass aggregate that can be three inches long containing 2,000 or more eggs.

<u>L.scutulata</u>

Egg laying of <u>L.scutulata</u> was observed at the Friday Harbor Laboratories from May through August, 1971, 1972, 1973.

The capsule is shaped like an inverted saucer with a diameter of 840um and usually contains from one to six zygotes (PLATES 2D, 43). The zygotes are yellow-orange in colour and about 100um in diameter. Within the capsule each zygote is enclosed in a relatively rigid egg covering. The zygotes are surrounded by a transparent fluid in the lumen

of the capsule. No albumen inside the egg covering was observed(PLATE 42D).

Three females, kept in separate dishes at a temperature of 10-14°C, produced 896, 1,034, and 1,398 egg capsules, respectively, within a period of 14 days in June, 1970. Egg capsules produced during the first week contained four or more eggs and the number of eggs reduced to three during the last four days. All three snails stopped production of capsules after 14 days (F-S. Chia, 1970-1974).

From monthly studies of the gonadal growth and maturation of gametes throughout one year, together with direct observations on spawning, it is concluded that the spawning season for the population at San Juan island is from May to September with the peak in July.

PART 2. DEVELOPMENT

L.sitkana

Fertilisation occurs at the primary oocyte stage and the germinal vesicle breaks down after egg deposition. Just prior to the emission of each polar body the egg membrane becomes more flexible and the shape of the egg changes, usually from spherical to an ovoid form. When the first polar body is released a slight indentation is produced at the animal pole, and during the second maturation division a small cytoplasmic extrusion is usually seen at the animal

pole from which the second polar body is emitted.

A small polar lobe is formed prior to each of the first four cleavages (PLATE 45 A, B, E) and may also be present in later stages. Before the first cleavage and during the formation of the first polar lobe a fertilisation membrane (vitelline membrane) is formed (PLATE 45A). This membrane gradually becomes separated from the whole embryo.

Early cleavage is spiral and follows the "typical" molluscan pattern (PLATE 45A-F). The eggs are sequentially fertilised such that the first part of the egg mass to leave the gonopore develops slightly in advance of the last part to leave. The difference is 3.5 to 4.5 hours which is approximately the time it takes to deposit the egg mass. These data support the idea that a precise site for fertilisation exists.

Blastula formation is complete 30 hours after fertilisation and gastrulation occurs about the third day. The stomodaeum which may be seen in PLATE 46A, usually forms between the third and fourth days. The embryo enlarges at the blastula stage, probably due to hydration, but there is no further growth until the velum is formed about the sixth day (PLATE 46B). At this time the young veliger ruptures the fertilisation membrane and begins to eat the albumen supply. During this phase the thrashing velar cilia break through both egg membrane and albumen layer so that the veliger comes to lie between the albumen and the capsule wall (PLATE

46C, D). The albumen is dispersed by the beating cilia and gradually consumed over a period of ten days, during which growth is rapid. At the end of the week the larval shell forms (PLATE 46E) and subsequently, owing to the "tanning" of the shell, the whole egg mass undergoes a series of visible colour changes from pale yellow, through pink, to dark brown.

In the laboratory, development from egg-laying to hatching takes 30 days at 10-11°C. However, developmental rate changes markedly with temperature (TABLE 2).

Hatching

The pre-hatching juvenile, after ingestion of essentially all of the albumen, almost fills the capsule (PLATE 46F). At this stage the velum is undergoing resorption and the snail moves around within the capsule by creeping along its inner surface. These movements of the foot gradually draw away the egg covering from the capsule wall and stretch it (PLATE 47A). This phase is characterised by repeated vigorous retractions of the columellar muscle which has the effect of jerking the shell over the animal's body and rasping the outer surface of the shell against the egg covering and capsule wall. Eventually the egg covering is stretched so much that it hangs in loose folds about the snail. It is interesting that scanning electron micrographs show rows of small tubercles on the surface of the larval shell, which disappear in the adult, probably due to erosion

(PLATE 49A).

In the next phase the foot repeatedly expands and contracts which exerts pressure on the capsule wall in two places; the ventral surface of the foot, and the dorsal surface of the shell. During this phase one sometimes sees the radula rasping on the loose folds of the egg covering.

The repeated exertion of pressure on the capsule wall eventually causes it to rupture in one place. If the egg covering has also broken, the snail eventually succeeds in pushing its tentacles through the crack. The foot creeps through the hole and pulls on the outside surface of the capsule to draw the shell and visceral mass out after it. The shell is invariably much larger than the size of the initial fracture and hence the pull of the foot gradually increases until suddenly the capsule wall bursts open and the snail crawls out (PLATE 47A-F). Sometimes when hatching is prolonged, part of the capsule wall becomes noticeably more flexible and appears to be dissolved in places, which may be due to the secretion of a hatching enzyme. At this stage the juvenile stretches the capsule into odd shapes before finally emerging (PLATE 48A-F).

After hatching the juvenile crawls out and feeds on the jelly of the egg mass and also the diatoms adhering to it. This eventually reduces the amount of jelly surrounding unhatched snails deeper in the egg mass, and facilitates their escape from the mass upon hatching.

Embryonic and juvenile growth

The growth of L.sitkana was recorded at 10-11°C in the laboratory and the results are shown in PLATE 49B.

There is an initial increase in size from 175um to 200um due to hydration during the early cleavage stages. A further increase occurs in the 6-day embryo when the veliger breaks through the fertilisation membrane and begins feeding on the albumen. This usually continues until the 25th day when growth becomes minimal. The juvenile is about 575um on hatching and it continues to grow at an ever increasing rate for at least two months, after which growth rate slows down. In the first year it can grow to 1 cm and attain a maximum size of 2.5 cm in its life span of two years.

L.scutulata

The eggs are at the primary oocyte stage when first laid. Polar bodies are formed 2 to 3 hours after laying and subsequently the first two cleavages occur between 6 and 10 hours, forming four blastomeres of approximately equal size (PLATE 50A, B). The third cleavage is unequal but synchronous, resulting in four micromeres placed spirally above four macromeres (PLATE 50C). Further cleavages form a coeloblastula without noticeably increasing the size of the embryo. Gastrulation takes place on the second and third days. The blastopore is shallow and irregular in outline (PLATE 50D). No cilia were evident at this stage.

Accompanying the development of the velum and its cilia, the egg membrane expands considerably, due partly to the young veliger's active movements (PLATE 50E). Further development of the foot, statocyst, larval shell, visceral mass and eye spot resembles that of <u>Bembicium nanum</u> (Littorinidae) which has been described in detail by Bedford (1966).

<u>Hatching</u>

The veliger becomes very active close to hatching and swims around inside the egg covering. The egg covering increases in diameter and accommodates these movements until the veliger is fully grown when it measures 160um (PLATE 50F).

The process of hatching is very rapid compared to that of <u>L.sitkana</u> and takes only about 20 seconds. It is composed of two phases; the first phase involves the rupture of the egg covering enclosing one of the veligers. This veliger thrashes around vigorously inside the capsule and usually causes all the other egg coverings to break. The second phase occurs almost immediately, when a hole is burst in the "lid" of the capsule and all the veligers quickly, but awkwardly, swim through it. The development from freshly laid eggs through hatching takes 7-8 days in the temperature range 10-14°C, and only 3 days at 22°C. The chronological events are summarised in TABLE 3.

Immediately after hatching the veligers begin to feed on diatoms and dinoflagellates, which can be followed clearly under a compound microscope. The length of the larval shell increases steadily during the following weeks, providing there is plenty of food in the culture dishes. After about three weeks of planktonic existence, most of the larvae have grown to 300-360um in shell length. At this stage they are lying on the substratum with much reduced velum, fairly well-developed tentacles, and a brownish coloured shell. Attempts in 1970, 1971 and 1973 to rear the larvae to metamorphosis failed. Some larvae survived a maximum of 25 days but all died before the completion of metamorphosis.

DISCUSSION

Oviposition and Larval Development

Some aspects of the reproduction of L.scutulata have been studied by Imai (1964, unpublished student report, Hopkins Marine Station, California) but his observations are very different from ours. For example, Imai reports that the egg capsules are held together by a mucus matrix which attaches to the substratum and that none of the egg capsules contain more than one egg. Further, the snails produce only one egg mass after each copulation and the egg diameter is 80um By contrast, all the egg capsules we observed were planktonic, never attached to each other, and contained an average of four eggs each. We also observed that an isolated female will continue to produce eggs up to 14 days after copulation and the egg diameter is 100um Owing to these and other disparities we suspect that either we are describing two separate species, or subspecies, or else the modes of reproduction of the two populations are dramatically different.

It should be stressed that L.sitkana and L.scutulata, two closely related species existing sympatrically for much of their distribution (L.scutulata from 58°N to 29°N; L.sitkana from 47° N to 56° N on the west coast), have adopted diverse modes of development, planktotrophic in L.scutulata and lecithotrophic in L.sitkana. According to a

classical hypothesis, direct (lecithotrophic) development, as illustrated by L.sitkana, is specifically adapted to polar and deep seas where larval food (diatoms) is scarce (Thorson 1936, 1950); but this hypothesis does not apply to the present case. An alternative hypothesis explaining the adaptive significance of the two patterns of development in related species of the same habitat has been advanced by Chia (1970) who argues that lecithotrophic development requires less energy in reproduction than that of planktotrophic development, and hence it is cheaper to operate in achieving the same purpose of maintaining a steady-state population. Careful study of the energy budget of these two species would test the validity of this hypothesis.

The formation of the small polar lobe during cleavage in L.sitkana is different from that of other molluscs such as Bithynia tentaculata studied by Hess (1971) and Bursa corrugata by D'Asaro (1969), because it is not only extruded at the 2-cell and 4-cell stages but also at the 8-cell, 16-cell and 32-cell stages. It is possible that it continues to form in later cleavages but it is difficult to observe after the 32-cell stage.

It was noted, page 94, that the egg capsule of both species gets progressively weaker throughout its existence until hatching occurs. This phenomenon has been reported by several authors and a review of the hatching process was

published by Davis (1967). The general idea is that a hatching enzyme is involved which causes preliminary softening of the capsule wall and/or egg covering.

To my knowledge a hatching enzyme has never been isolated from molluscan larvae. However my observations indicate that: (a) At a certain stage in development it suddenly becomes very much easier for the juvenile/veliger to break out from the capsule. This together with the fact that much of the egg capsule and all the egg covering are dissolved soon after hatching, indicates the release of some substance that mediates hatching; (b) The rate of capsule softening is temperature dependent, being more rapid at higher temperatures, leading to shorter hatching times.

The jelly envelope of L.sitkana egg masses also degenerates with time, which may be another effect of the hypothetical hatching enzyme. However the jelly may also age naturally, or be progressively destroyed by other organisms including; adult snails, juvenile snails, nematodes, crustaceans and bacteria.

The fact that unicellular algae and diatoms readily adhere to the jelly of the egg mass may be considered as a secondary function of the jelly , as this complex can serve as food for both juveniles and adults. Moreover, these diatoms may serve to provide extra oxygen for the developing embryos embedded in the jelly in turn receiving a higher concentration of carbon dioxide compared to that in the

surrounding water, owing to the relatively high activity of the juveniles (see APPENDIX 1).

The possible role of the tubercles on the larval shell in aiding the hatching process, described in this chapter, is important as it suggests a functional significance of these structures which have also been noted in Rissoa sarsi by Fretter (1971).

It should be mentioned that contrary to a statement made in a previous paper (Buckland-Nicks, Chia and Behrens, 1973), I have since found evidence from cine films of hatching that the radula may be used actively by juvenile L.sitkana during the final stages of hatching, to rasp through the egg covering and perhaps also the softened capsule wall.

In L.scutulata the egg covering gradually expands in size from the early veliger stage. Linke (1933) suggested that this was due to a change in the osmotic pressure of the fluid surrounding the veliger in Littorina littorea . My observations tend to support the idea of a stretching of the membrane by mechanical means, such as the thrashing movements of the velar cilia. However, the osmotic changes in the fluid within the egg covering have not been measured and this may be an additional factor.

Factors Affecting Breeding Seasons

The breeding season of *L.sitkana* is biannual with peaks occurring in April and September, whereas that of *L.scutulata* is annual with the peak in July. This pattern of breeding activity has been observed for three years which indicates, as is the case with many other invertebrates, that the snails are monitoring some consistent environmental variable. It is likely that there are also short term factors which control the times of copulation and spawning within a reproductively favourable period.

Long term factors :

1. Food supply
2. Temperature
3. Photoperiodicity
4. Tide level

Each of these will be considered in turn and their possible influence on the breeding of *L.sitkana* and *L.scutulata* :

1. Food supply

Although the adult snails of both species feed on similar kinds of algae and diatoms, the veligers do not. The veliger of *L.sitkana* is well supplied with albumen for development to the juvenile stage within the egg capsule. However, the veliger of *L.scutulata* is dependent on the planktonic food, such as diatoms. Plankton blooms occur bi-annually in the Spring and late Summer in

the vicinity of San Juan island which partly correlates with the summer breeding of L.scutulata.

2. Temperature and tide level

The sea water temperature throughout the year is fairly constant around San Juan island at about 9°C (48°F). This is not true for the intertidal tide pools, in which temperatures vary from 5°C (41°F) in winter, to 30°C (86°F) in summer. The latter is partly due to the increased temperature and partly to the much longer exposure times between tides at this time of year. In April, when exposure is maximal, temperature is seldom a limiting factor.

These data correlate with the breeding of L.sitkana whose egg masses are easily dessicated when exposed for long periods at relatively high temperatures (Behrens 1970). However, this does not affect breeding in L.scutulata whose planktonic egg capsules float out with the tide.

The increased temperature , itself, does not decrease hatching success (see APPENDIX 1) but coupled with long exposure times it does, through desiccation . Another factor accompanying desiccation is increased salinity which may also be important under these circumstances.

3. Photoperiodicity

This is the most consistent

environmental variable and for this reason is worthy of consideration.

The day length changes throughout the year such that the longest days are in July and the shortest in December. However a plot of the rate of change (daily increment) of day length shows two peaks: One in March/April and the other in September/October. It is interesting that if maximal temperatures are plotted throughout the year one observes a peak in summer and two periods (Spring, Fall) when the temperature is mild. For three years the breeding season of L.sitkana has followed the double peak pattern closely (PLATE 44).

L.scutulata spawns in one season beginning in May with the peak in July and terminating in August, which is similar to the plot for increasing day length.

Studies of nutritional storage in Littorina littorea from the east coast of England over a 1 year period, showed two unequal peaks for lipid levels in both male and female snails; the smaller peak occurred at the end of February and the main peak was around September (Williams, 1970). Carbohydrate levels showed similar fluctuations with peaks in January and August. This fluctuation of nutritional storage compares well with observations on the annual fluctuation of egg production in L.sitkana. Therefore egg production might have resulted from a state of maximum nutritional storage. But as has been mentioned earlier the

peaks of oviposition coincided also with those for photoperiodicity. Thus, biological events such as nutritional storage and egg production may be under the influence of the most consistent environmental factor, photoperiodicity.

This line of argument is supported by the work of Meenakshi and Scheer (1969), who studied the regulation of galactogen synthesis in the slug, *Ariolimax columbianus*. They concluded, "Present evidence suggests that growth of the albumen gland is under endocrine control from a centre in the optic tentacle, and that endocrine effects on synthesis of galactogen are a consequence of the effect on growth."

Conclusions :

Long term factors which may have a direct effect on breeding are food supply and desiccation (temp./tide level). However both these variables correlate with photoperiodicity. It is possible that the snails in some way monitor day length changes and so take advantage of optimal breeding conditions.

Short term factors :

1. Trematode parasites
2. The sulphide system
3. Wave action

4. Predators

5. Pollution

1. Trematode Parasites

Parasitised snails are usually weaker than healthy animals and are often found lying on the substratum without the strength to 'right' themselves. These snails are more susceptible to predation by shore crabs and hermit crabs. The crabs place one cheliped between the operculum and the foot, preventing shell closure, and then pick out the snail bit by bit with the other cheliped. In this way the parasites reach their second intermediate hosts, in which they form metacercariae. Previous studies on trematode parasites of these snails were conducted by Ching (1962, 1963 a, b).

One often finds an entire tide-pool of shells of both littorines occupied by hermit crabs. This can be as many as 600 individuals. In these situations it is difficult to find a healthy snail. However one might find a neighbouring tide pool with few or no hermit crabs and all healthy snails. This indicates a patchy distribution of the parasites which probably reflects a low infection rate of the primary host and possibly its feeding habits.

A heavily infected snail is incapable of forming an egg mass because both gonad and glandular elements are reduced to a minimum size.

In 1972 one population of L.scutulata at False Bay was 60% parasitised in May. Only six weeks later the same population was reproductively active and few parasitised individuals were found. This evidence supports the idea of a seasonal cycle for the parasites in Littorina, which has been proposed by Robson and Williams (1971).

2. The Sulphide System

The sulphide system was studied by Fenckel and Reed (1970) who noted that the environment became virtually anaerobic in certain situations.

At False Bay one often encounters black sand, decaying vegetation, a smell of hydrogen sulphide and a black coating on the underside of rocks. In tide pool situations, these conditions are unfavourable for the mating and breeding of snails. Egg masses deposited by L.sitkana in areas of 'clean sand', which later underwent degradation to black sand, failed to hatch out (see APPENDIX 1). Adult snails collected from these tide pools had immature gonads and males often lacked a penis.

In one area of False Bay breeding of L.sitkana was delayed by two months in 1971. Breeding began three weeks after the decaying vegetation and black sand had subsided.

3, 4. Wave Action and Predation

Excessive wave action will prevent snails

from copulating, or disturb them while in the process, which might cause the spermatophore to be lost in the water. Similarly a predator such as Hemigrapsis nudus or a sea-bird may disturb copulating snails, with the same result.

However, splashing can also stimulate either species to copulate It is not known to what extent these two factors can affect breeding success.

5. Pollution

The effects of oil on the survival of some marine larvae has been studied by Chia (1970, 1971a, 1973) and Woodin et al. (1972), who found that, in general, mortality was high. They considered that species with planktonic larvae would have a better chance of surviving in an area compared to species with benthic larvae, for though the local population might be killed off, recruitment occurs fairly readily once the oil has diminished. This would apply to Littorina scutulata ; however populations of L.sitkana might be affected more drastically, since the benthic development of this species would make the larvae susceptible to the poisonous effects of the oil. In the event of an oil spill occurring at the beginning of the breeding season one could expect considerable reduction in both fecundity and survival of juveniles, in this species.

Conclusions :

1. Heavily parasitised snails cannot reproduce.

2. Sometimes infection reaches 60% or more.

3. If this level of infection were maintained in *Littorina* populations, it would drastically reduce fecundity.

4. Evidence indicates a seasonal cycle of parasites in *L.scutulata* that ends at the beginning of the breeding season.

5. The sulphide system, which is characterised by anaerobiosis, hydrogen sulphide gas and decaying vegetation, has deleterious effects on the reproductive habits of *L.sitkana* (it is assumed that *L.scutulata* would be affected similarly): Embryos die either through lack of oxygen, or the presence of toxic chemicals in the water, or because of bacteria, or any combination of these factors.

TABLES, PLATES AND FIGURE LEGENDS

TABLES, PLATES AND FIGURE LEGENDS

TABLE 1

Staining Reactions of the Female Reproductive Systems and their Secretions

SECRETIONS STAINED:

STRONG REACTION +++, MODERATE R. ++, WEAK R. +, NEGATIVE R. -

SPECIES	STAIN	YOLK	EGG MEMBRANE	ALBUMEN	EGG COVERING	CAPSULE FIBRES	CAPSULE WALL	JELLY ENVELOPE
Littornia sitkana	AB/AY	+ BLUE	+ BLUE	++ BLUE-GREEN	+++ YELLOW	/	+++ YELLOW	+++ GREEN-BLUE
	MASSON'S TRICHROME	+++ RED	-	+++ BLUE	-	/	+ BLUE ++ RED	-
Littorina scutulata	AB/AY	+ BLUE	+ BLUE	/	+++ YELLOW	+++ YELLOW	+++ YELLOW	/
	MASSON'S TRICHROME	+++ RED	-	/	-	-	+ BLUE	/

TABLE 2
Chronology of Development of L. sitkana

STAGE OF DEVELOPMENT	TIMES AT DIFFERENT TEMPERATURES		
	9°	10-11°	12-13.5°
Oviposition: Fertilization of first egg of the egg mass	0 hr 0 min	0 hr 0 min	0 hr 0 min
Fertilization of last egg of the egg mass	4 hr 30 min	4 hr 30 min	4 hr 30 min
1st polar body forms		7 hr 05 min	6 hr 35 min
2nd polar body forms		8 hr 50 min	8 hr 25 min
2-cell stage		9 hr 15 min	8 hr 45 min
4-cell stage		13 hr 25 min	12 hr 05 min
8-cell stage	20 hr	16 hr 55 min	13 hr 20 min
16-cell stage		20 hr 35 min	16 hr 20 min
32-cell stage		23 hr 25 min	19 hr 35 min
Blastula	36 hr	30 hr	
Blastula-gastrula	2 1/2 days	2 days	
Gastrula	5 1/2 days	3 1/2 days	
Gastrula-veliger	9 days	6 1/2 days	
Veliger	12 1/2 days	7 1/2 days	
Late veliger	23 days	10 1/2 days	
Pre-hatching juvenile		21 days	
Juvenile (hatched from capsule)		30 days (all hatched)	

TABLE 3

Time Sequences for the Developmental Stages of Littorina scutulata

STAGE OF DEVELOPMENT	TIME
Egg laying	0 hours
Polar body formation	2-3 "
2-cell stage	6-8 "
4-cell stage	10 "
Late cleavage stages	24 "
Blastula	30 "
Gastrula	2-3 days
Young veliger	4 "
Pre-hatched veliger	6 "
Hatching	7-8 days at 13-15° C 3 days at room temperature (22° C)

TABLE 4

Hatching Success of Embryos Exposed to Different Environmental Conditions

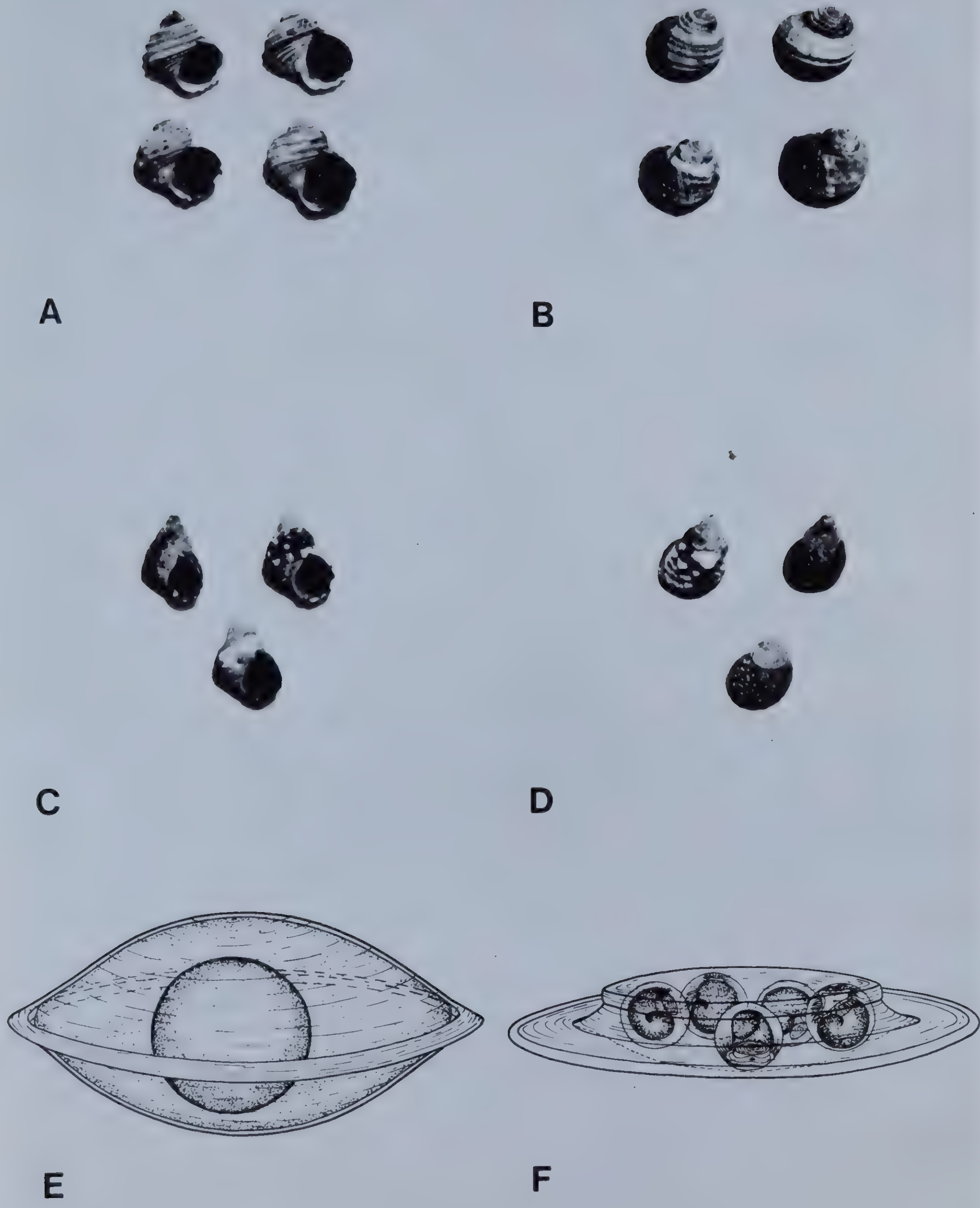
#	EXPERIMENT	TEMP. °C	INITIAL pOx (mm Hg)	# OF FERTILE EGGS AT START	# OF EMBRYOS HATCHED	% HATCHED
A	1 Egg Mass + Diatoms (C)	10	160	305	305	100
	2 Egg Mass - Diatoms (C)	10	160	330	330	100
	3 Egg Mass + Diatoms (E)	10	40	320	5	1.5
	4 Egg Mass - Diatoms (E)	10	40	339	0	0
B	5 Egg Mass - Diatoms (C)	10	160	306	306	100
	6 Egg Mass - Diatoms (E)	20	160	400	400	100
	7 Egg Mass - Diatoms (E)	25	160	350	345	98
	8 Egg Mass - Diatoms (E)	30	160	330	330	100
	9 Egg Mass - Diatoms (E)	32	160	300	300	100
	10 Egg Mass - Diatoms (E)	34	160	360	350	97
C	11 Egg Mass - Diatoms (C) (No Sand)	10	160	930	930	100
	12 Egg Mass - Diatoms (E) (Clean Sand)	10	160	907	15	1.6
	13 Egg Mass - Diatoms (E) (Black Sand)	10	160	869	869	0

PLATE 1

Photomontage of False Bay at low tide, San Juan island, showing the general distribution of tide pools and the two sites at which caged populations were maintained for 2 years (arrows).

PLATE 2

Figures A and B. Dorsal and ventral views of four different
Figure E. Egg capsule of L.sitkana, Figures C and D.
Dorsal and ventral views of three different colour
morphs of L.sitkana (actual size).
Figure E. Egg capsule of L.sitkana, x75.
Figure F. Egg capsule of L.scutulata, x95.


A
B
C
D
E
F

<u>PLATE 3</u>

<u>Figure</u> <u>A</u>. Diagram of apparatus used for vinyl acetate injection. Note: Snail dissection (D), showing micropipette (mp) entering anterior tip of reproductive system (rs); Micromanipulator (M), illustrating the position of the clamp (c) and glass-tubing sheath (gt); Injection apparatus (I), showing rubber bands (r) securing hypodermic syringe (s) to wooden mount, and a screw micro-adjuster (ma) for controlling the rate of flow of plastic.

<u>Figure</u> <u>B</u>. Plastic replica of the female reproductive system of <u>L.scutulata</u>, viewed from the right side. Note: The oviduct takes a spiral path through the covering gland (co) and a U-shaped path through the capsule gland (c), before opening into the vestibule (v), <u>x27</u>,

<u>Figure</u> <u>C</u>. Same as in B, viewed from a right, ventral aspect, <u>x27</u>.

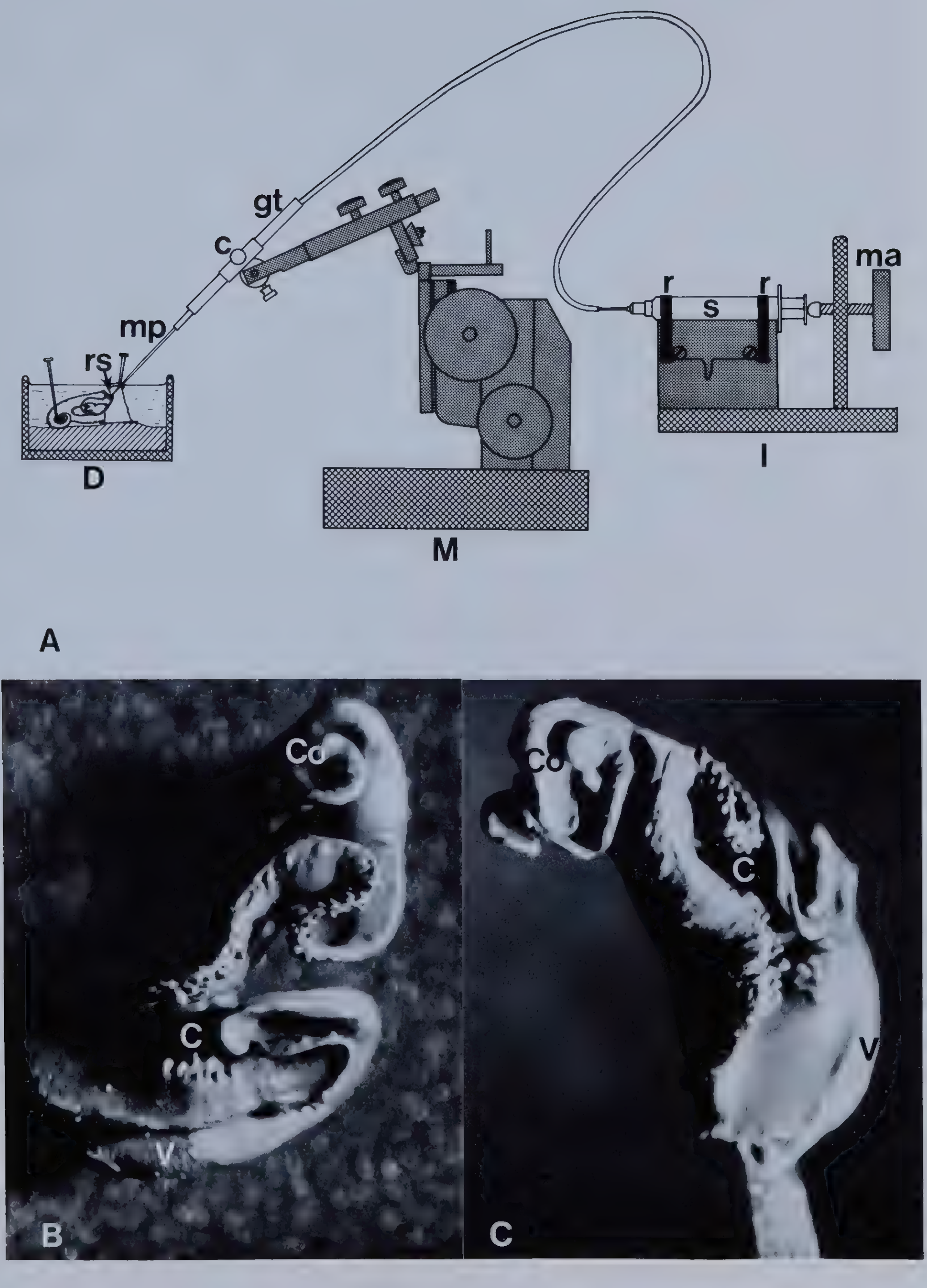

gt
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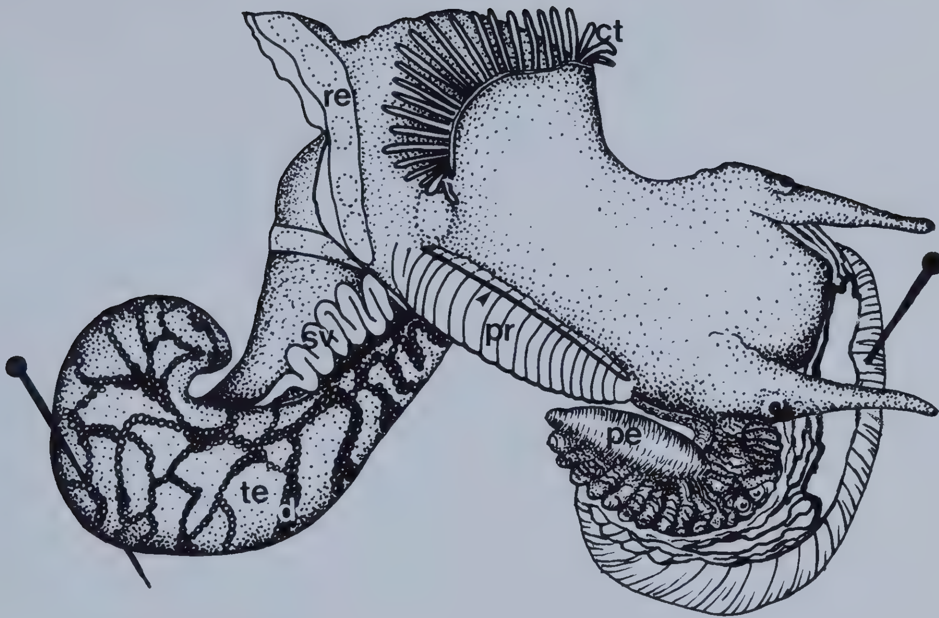
PLATE 4

Figure A. Sketch of dissected ripe male L.sitkana to show the general arrangement of reproductive organs in the body: Testis (te); digestive gland (d); seminal vesicles (sv); prostate gland (pr); rectum (re); penis (pe); ctenidium (ct). The prostate is open to the mantle cavity but its left lobe has a thickened edge which effects closure (arrow), x9.

Figure B. Sketch of unripe female L.sitkana removed from shell and viewed from the right side, to show the general arrangement of reproductive organs: ovary (ov); digestive gland (d); oviduct (o); receptaculum seminis (rs); albumen gland (a); capsule gland (c); jelly gland (j); vestibule (v); hypobranchial gland (h); kidney (k), x9.



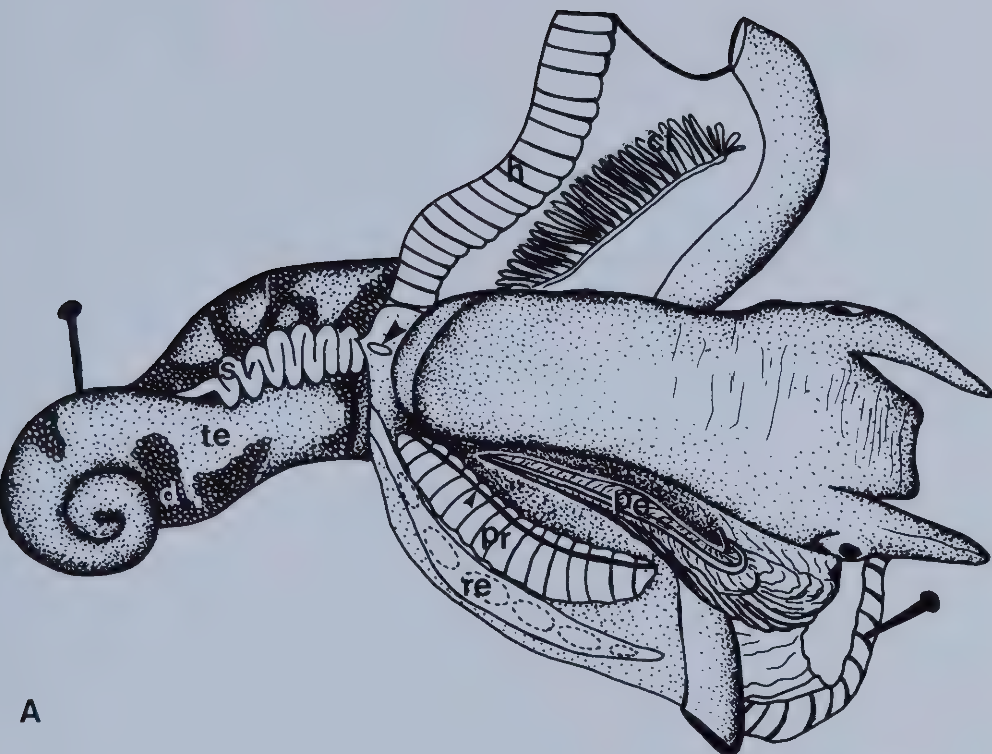
A

B

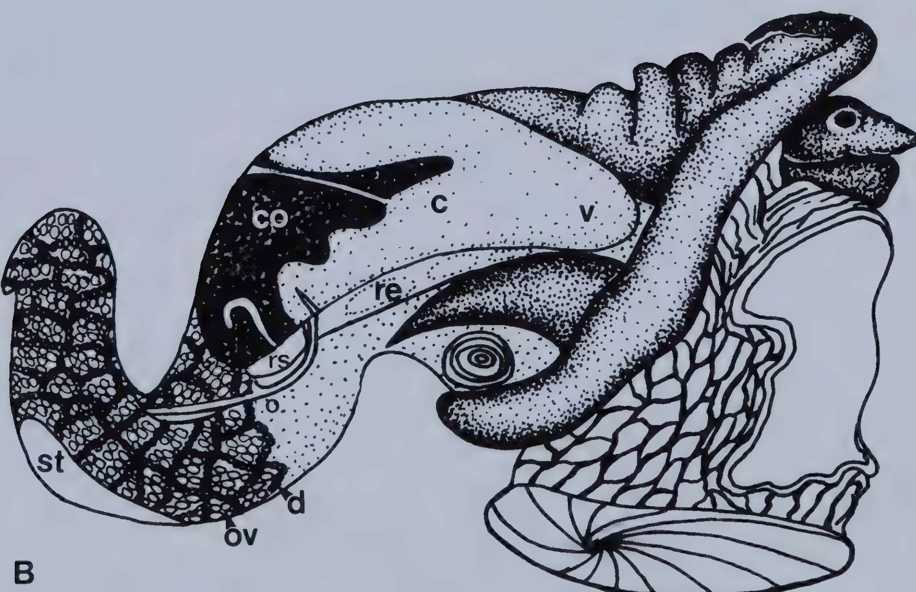
PLATE 5

Figure A. Sketch of dissected ripe male *L.scutulata* to show the general arrangement of reproductive organs in the body: testis (te); digestive gland (d); seminal vesicles (sv); prostate gland (pr); rectum (re); penis (pe); ctenidium (ct); hypobranchial gland (h). The prostate is open to the mantle cavity but its left lobe has a thickened edge which effects closure (arrow), x9.

Figure B. Sketch of ripe female *L.scutulata* removed from shell and viewed from the right side, to show the general arrangement of reproductive organs: ovary (ov); digestive gland (d); oviduct (o); receptaculum seminis (rs); covering gland (co); capsule gland (c); vestibule (v); kidney not visible, x9.



A



B

PLATE 6

Figure A. Light micrograph of right lobe of prostate gland of *L.sitkana*, showing gland cells containing large secretion droplets (arrow), interspersed by ciliated cells, x500. ,

Figure B. Same as A, showing fine secretion droplets (arrow), x500.

Figure C. Low power electron micrograph of penial gland [*L.sitkana*], showing heterogeneous secretion granules (s) passing out of the gland cell process into the lumen (lu). Micro- and macroapocrine secretions can be seen budding off from cells, or free in the lumen (arrows), x6,500.

Figure D. Low power electron micrograph of penial gland [*L.sitkana*], showing dense muscle layer (m), through which gland cell processes have passed to empty in the lumen at lower left. Heterogeneous secretion granules are visible in two processes (s). Inset : Gland cell processes passing through muscle layer, x6,500 (both figures).

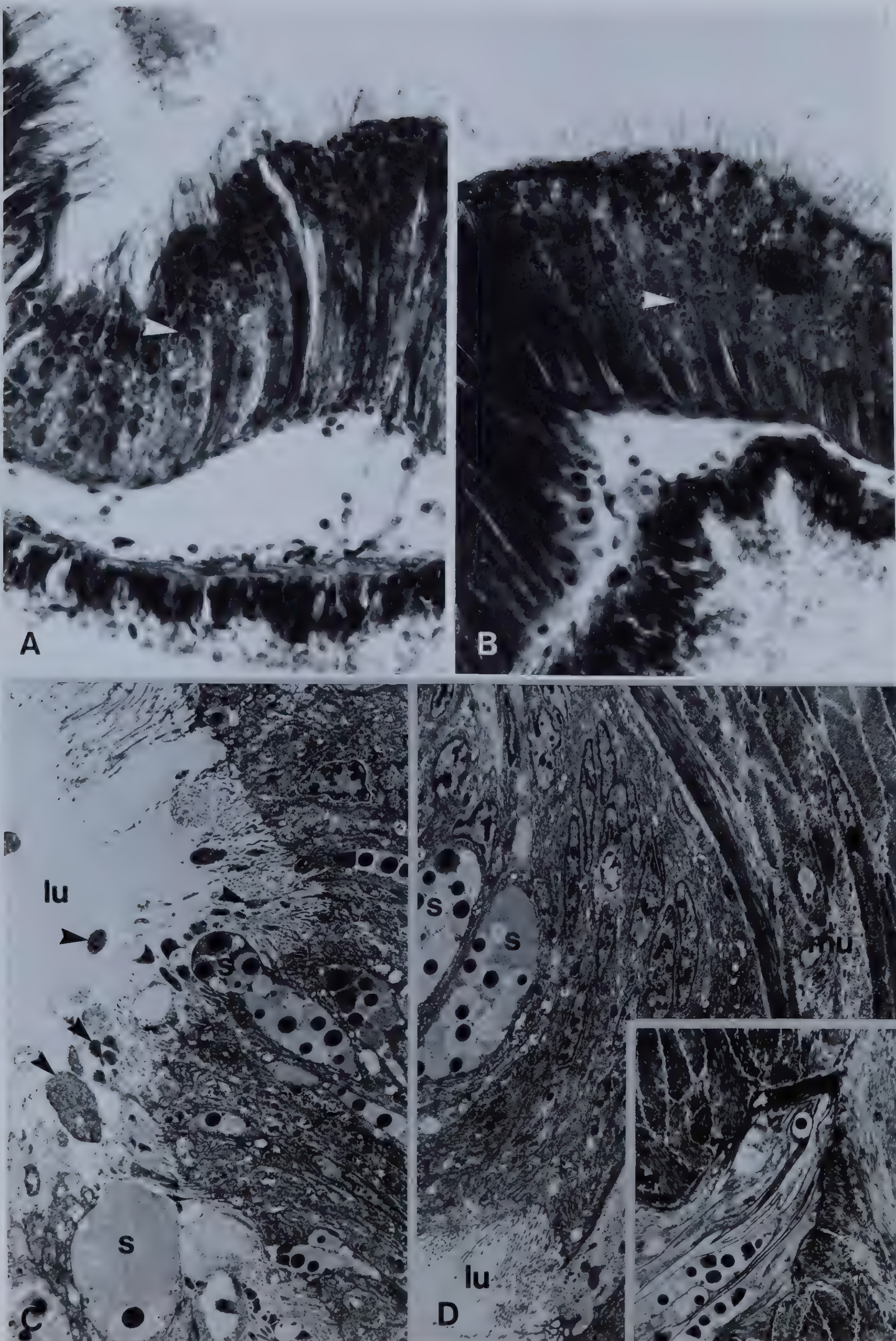

A
B
lu
s
s
C
s
s
mu
lu
D

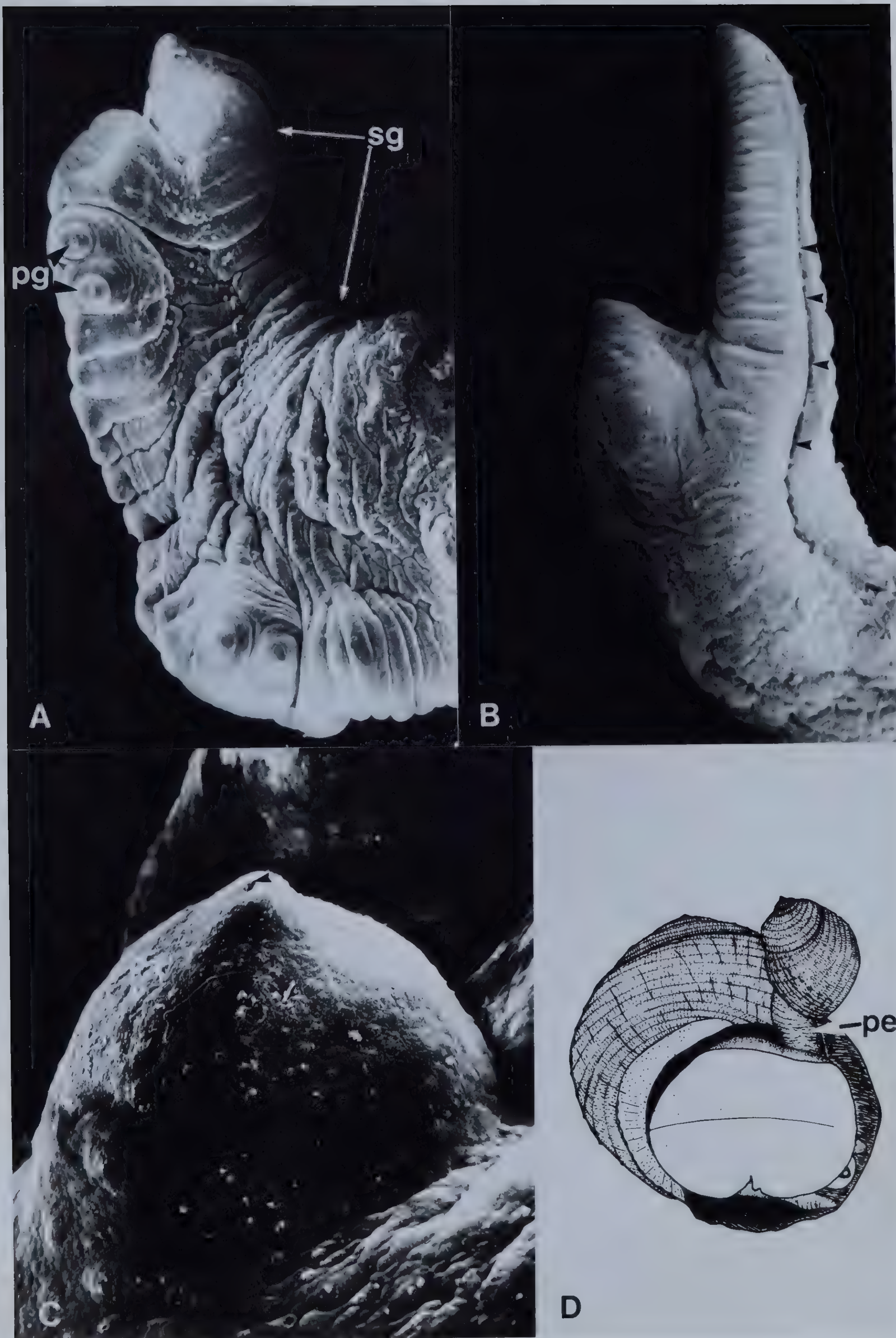
PLATE 7

Figure A. Scanning electron micrograph of the penis of *L. sitkana*. Note: sperm groove in dorsal furca (sg); small bifurcation at tip of penis; and 14 penial glands (pg), x40.

Figure B. Scanning electron micrograph of the penis of *L. scutulata*. Note: sperm groove in elongate, dorsal furca (arrows), x145.

Figure C. Scanning electron micrograph of penial gland [*L. sitkana*]. Note: Pore (arrow), x800.

Figure D. Diagram of copulating snails [*L. sitkana*]. Dimorphism, such as shown here, is often observed but may not be a general rule. Note: Penis (p) inserted in mantle cavity on right side of female, x3.


sg
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D

PLATE 8

<u>Figure</u> <u>A</u>. Early spermatogonium. Note: nuclei (n), each with large nucleolus, and golgi body (g). Mitochondria and dense granules are also visible, <u>x7,500</u>.

<u>Figure</u> <u>B</u>. Late spermatogonium (metaphase). The chromosomal material is lined up along the axis of the cell, prior to cell division, <u>x9,000</u>.

<u>Figure</u> <u>C</u>. Primary spermatocyte (zygotene). Note: nucleus (n) with diffuse chromatin and large mitochondrion (m). Dense granules are visible at upper left, <u>x10,000</u>.

<u>Figure</u> <u>D</u>. Primary spermatocyte (pachytene). Note: aggregations of chromatin prior to axial alignment in diplotene; nucleus (n), <u>x13,000</u>.

<u>Figure</u> <u>E</u>. Secondary spermatocyte showing patchy chromatin in nucleus (n) and several golgi bodies (g). <u>x10,000</u>.

<u>Figure</u> <u>F</u>. Stage A spermatid. Note: nucleus (n); mitochondria (m); and golgi body. Arrow indicates differentiated zone, at base of nucleus, <u>x23,000</u>.

All micrographs made from sections of mature testis of <u>L.sitkana</u>.

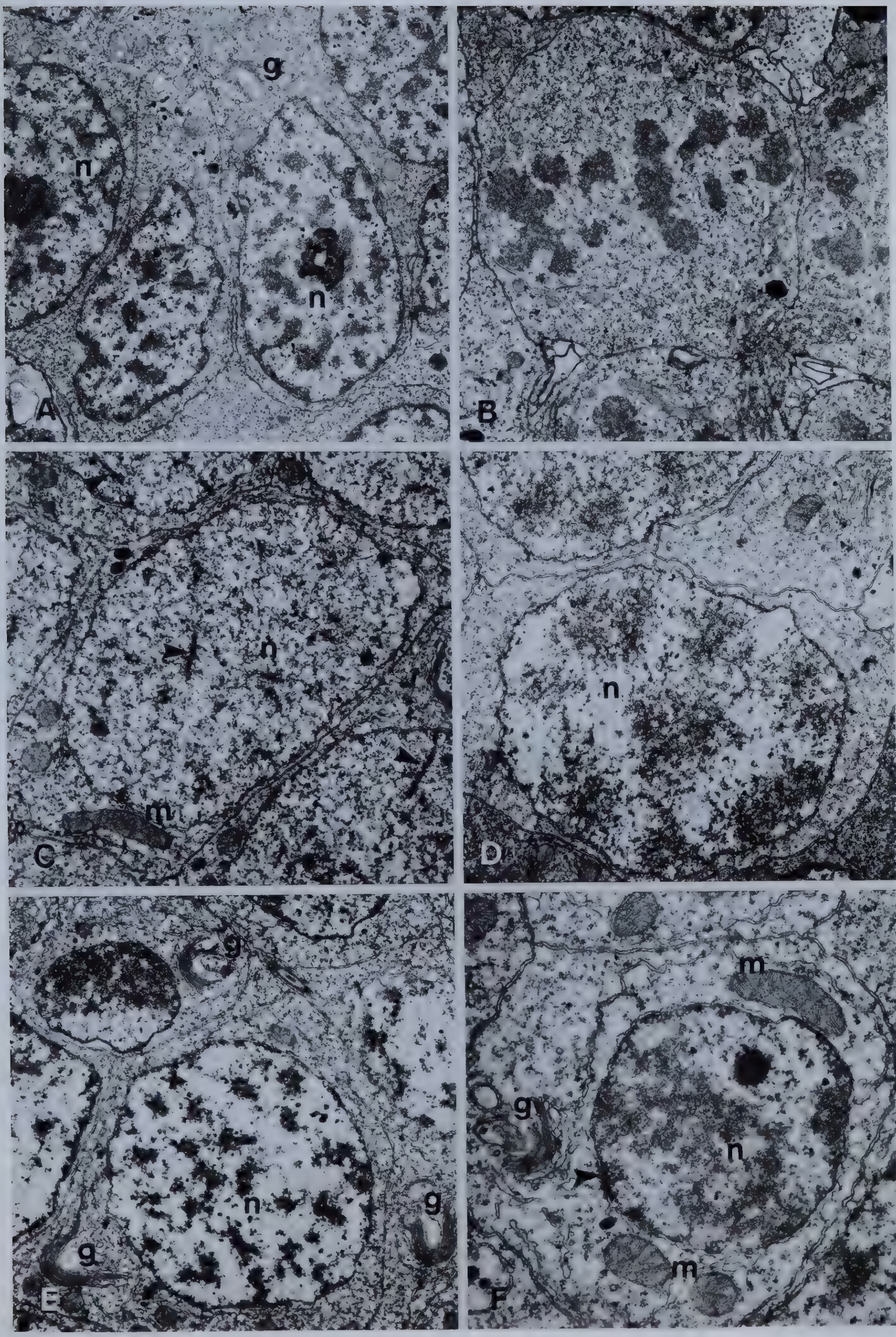

g
n
n
A
B
n
m
C
n
D
g
n
g
g
E
m
g
n
m
F

PLATE 9

Figure A. Type B spermatid. Distal centriole (d) attached to plasma membrane (arrow), together with proximal centriole (p), lie in basal invagination of nucleus (n). Note: dense material lining inner membrane at base (differentiated zone), and apex of nucleus, x25,000.

Figure B. Apical portion of type B spermatid showing acrosomal granule (ag) and interstitial granule (ig) in process of formation from golgi body (g), overlying apex of nucleus (n), x53,500.

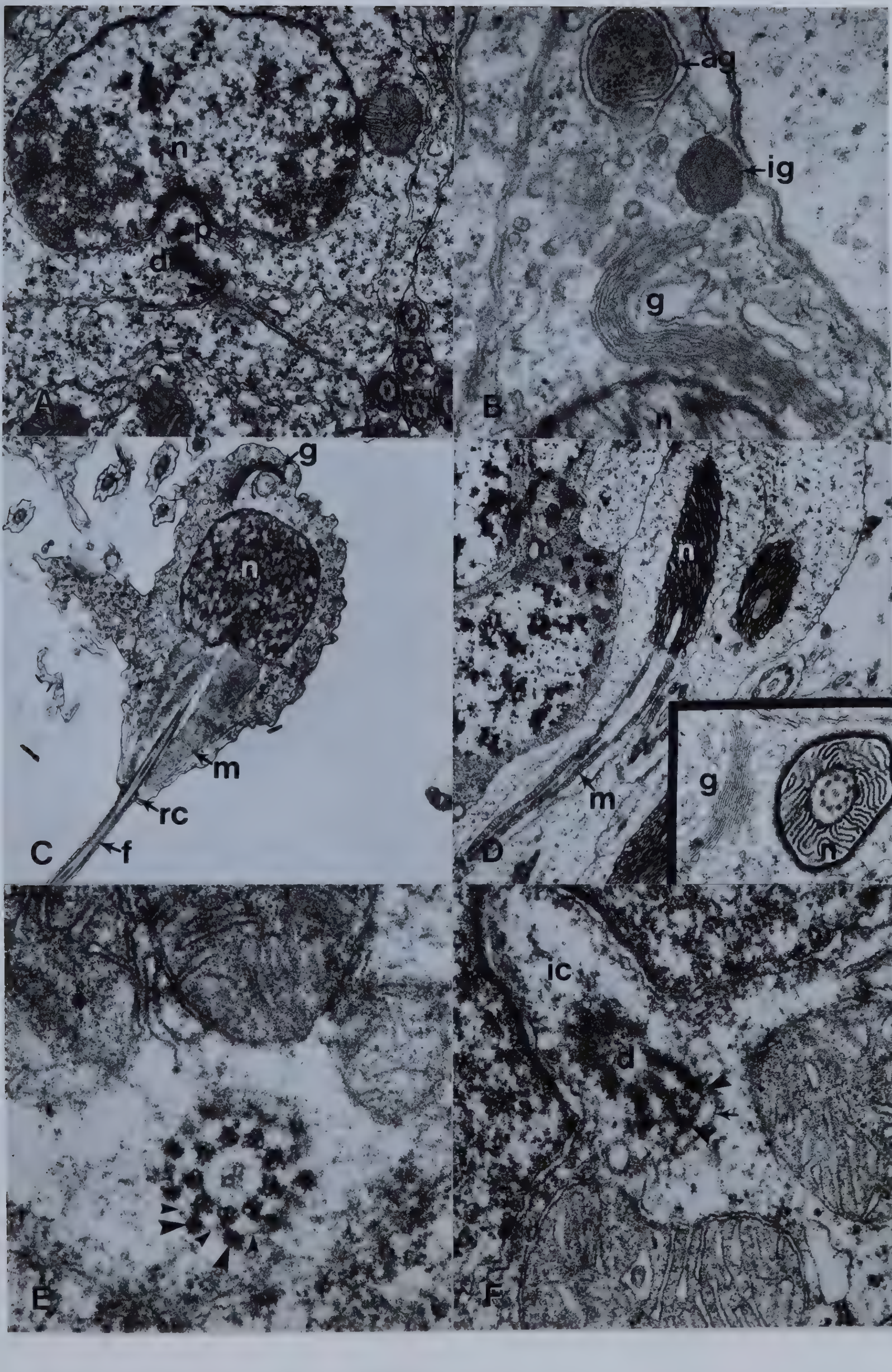
Figure C. Type C spermatid showing golgi body (g) overlying apex of nucleus (n), below which mitochondrial Nebenkerne (m) are in process of elongation along flagellum (f). Note: ring centriole (rc) at base of Nebenkerne, x10,000.

Figure D. Type D spermatid showing spiralled chromatin in nucleus (n) and very elongate Nebenkerne (m) x7,500. Inset : Golgi body (g) has migrated laterally from apex of nucleus. Condensation of chromatin in lamellar plates is visible within nucleus (n), x30,000.

Figure E. Transverse section (T-S) in base of distal centriole, showing cartwheel arrangement of dense projections. Note: Each dense projection (large arrows) is attached to a flagellar doublet by a thick strand of material and to the adjacent two projections by thin strands of material (small arrows), x90,000.

Figure F. Oblique grazing section through point of attachment (large arrows) of distal centriole (d) to invaginated plasma membrane (small arrow). Note: formation of intranuclear canal (ic), x71,000.

All micrographs made from sections of mature testis of L.sitkana.


n
p
d
A
ag
ig
g
B
n
g
n
m
rc
f
C
n
m
g
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E
ic
d
F

PLATE 10

Figure A. Oblique grazing section through distal centriole. Ring centriole (rc) still forms part of distal centriole (d) and is also attached to invaginated plasma membrane, which appears as an indistinct vesicle below it, x44,500.

Figure B. Slightly oblique, grazing section through distal centriole at point of attachment of ring centriole (rc), with invaginated plasma membrane (p), x55,500.

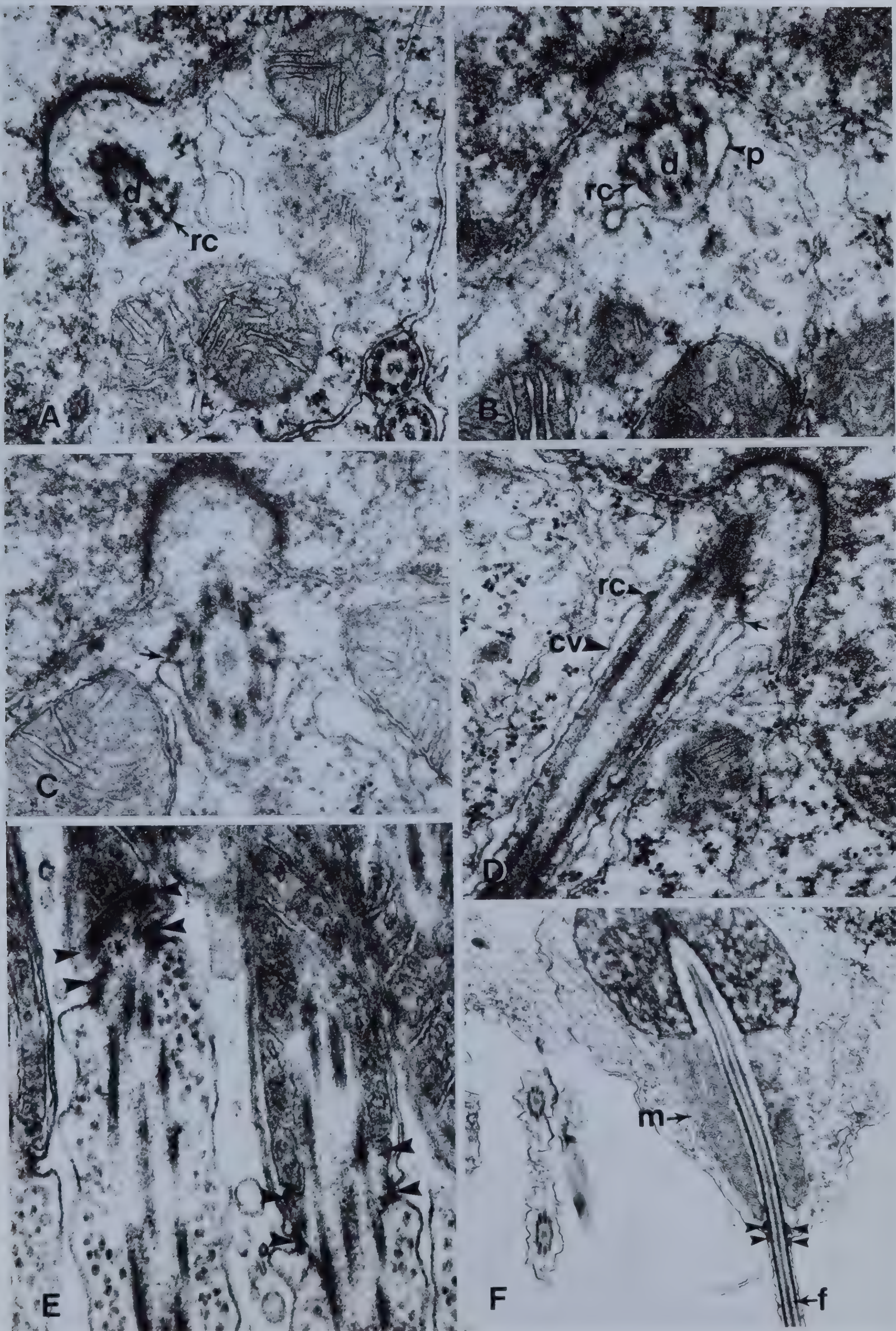
Figure C. Longitudinal section (L-S) through base of distal centriole, showing how dense projection attaches to invaginated plasma membrane (arrow), x66,700.

Figure D. L-S through cylindrical vesicle (cv), showing ring centriole (rc) in cross section on left and still attached to dense projection of distal centriole on right (arrow). At these points cylindrical vesicle is attached to ring centriole, x55,500.

Figure E. Longitudinal grazing sections through mitochondrial- tail junction, showing two dense spiral bands (upper left) and sections through similar bands (arrows), x66,700.

Figure F. L-S through stage C spermatid, showing ring centriole as two dense bands (arrows) during its posterior migration at base of Nebenkerne (m), x15,500.

All micrographs made from sections of mature testis of L.sitkana.


d
rc
A
d
p
rc
B
C
rc
cv
D
E
m
f
F

<u>PLATE 11</u>

<u>Figure</u> <u>A</u>. T-S through stage B spermatid. Procentriole (p) lies perpendicular to distal centriole (d), <u>x40,000</u>.

<u>Figure</u> <u>B</u>. L-S through stage B spermatid. Procentriole (p) is oriented obliquely to tip of distal centriole (d). Note: Dense material at tip of distal centriole, and centriolar 'foot' (arrow), <u>x40,000</u>.

<u>Figure</u> <u>C</u>. L-S through stage B spermatid. Procentriole (p) is aligned with distal centriole (d), <u>x40,000</u>.

<u>Figure</u> <u>D</u>. L-S through stage B spermatid. Procentriole (p) has become attached obliquely to distal centriole (d), <u>x30,000</u>.

<u>Figure</u> <u>E</u>. L-S through stage B spermatid. Procentriole (p) has fused coaxially with distal centriole (d). Note: dense plate beneath procentriole (basal body) {arrow}, <u>x70,000</u>.

<u>Figure</u> <u>F</u>. Formation of acrosomal granule (ag) from a golgi vesicle. Note: dense material at top of vesicle (arrow), <u>x80,000</u>.

All micrographs made from sections of mature testis of <u>L.sitkana</u>.

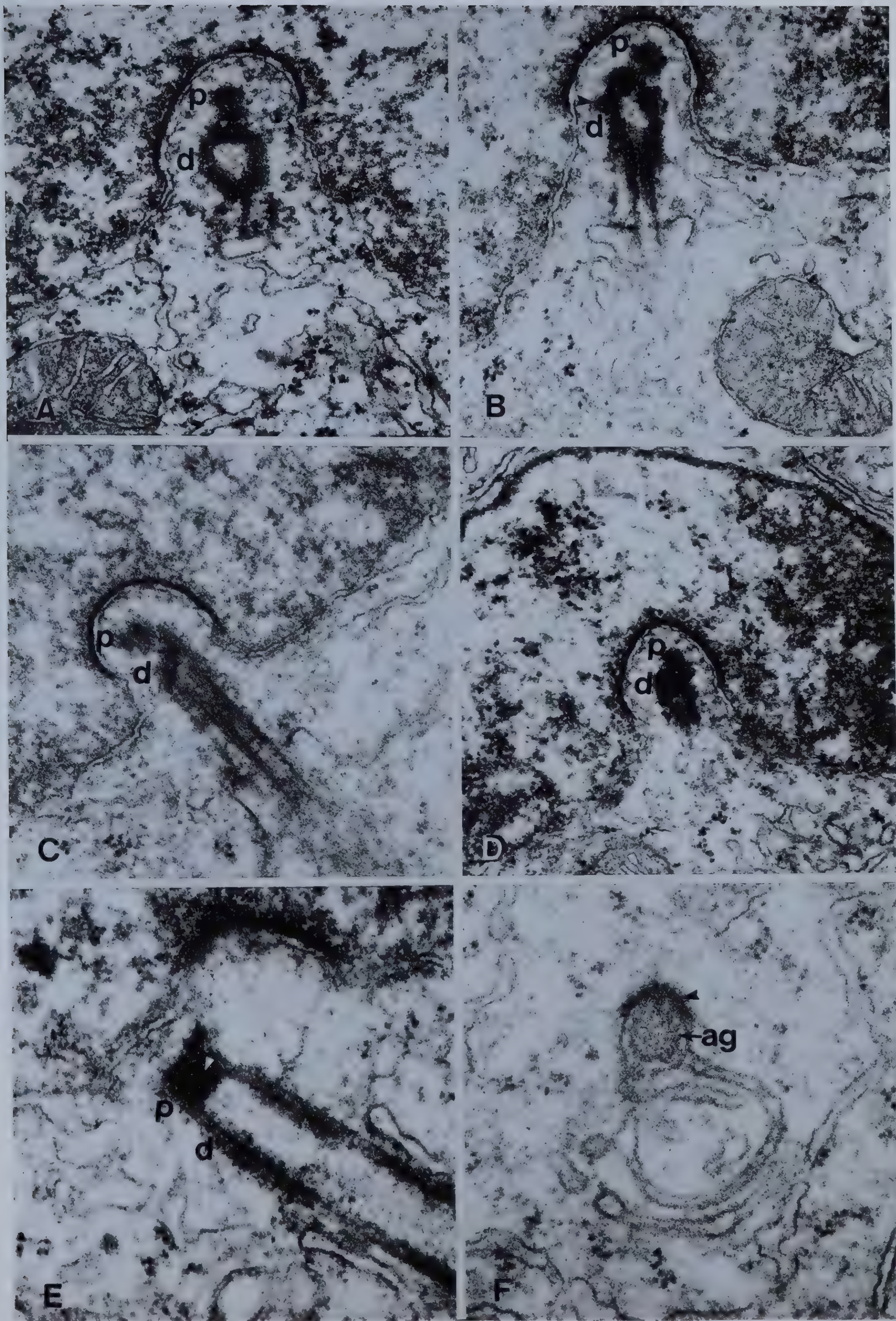

p
d
A
p
d
B
p
d
C
p
d
D
p
d
E
ag
F

PLATE 12

Figure A. Acrosomal granule (large arrow) overlies interstitial granule (small arrow) and nucleus at lower left. Golgi saccules are visible at top right, x90,000.

Figure B. L-S through developing acrosome. Note: acrosome development from golgi saccule (g); dense material on sides of acrosome (arrow); interstitial granule (ig) beneath acrosome; and dense matrix inside cone, which later forms acrosomal rod (ar), x70,000.

figure C. L-S through developing acrosome. Note: formation of acrosome cone (ac); interstitial granule (ig) in depression of nucleus (n); dense material on sides of acrosome (arrow); golgi body (g), x70,000.

Figure D. L-S through developing acrosome. Note: nearly complete acrosome cone (ac); disk-like interstitial granule in depression of nucleus (n); and golgi body (g) overlying acrosome, x28,000.

Figure E. L-S through developing acrosome in a plane perpendicular to that in Figure D. Note: hook-shaped acrosome and adjacent golgi body (g); interstitial granule (ig) overlying nucleus (n), x27,000.

Figure F. T-S through apex of nucleus and base of acrosome. Note: microtubules surround acrosome (arrows). microtubules are also visible in section through basal body (at centre) and nuclear region (top right), x67,000.

All micrographs made from sections of mature testis of L.sitkana.

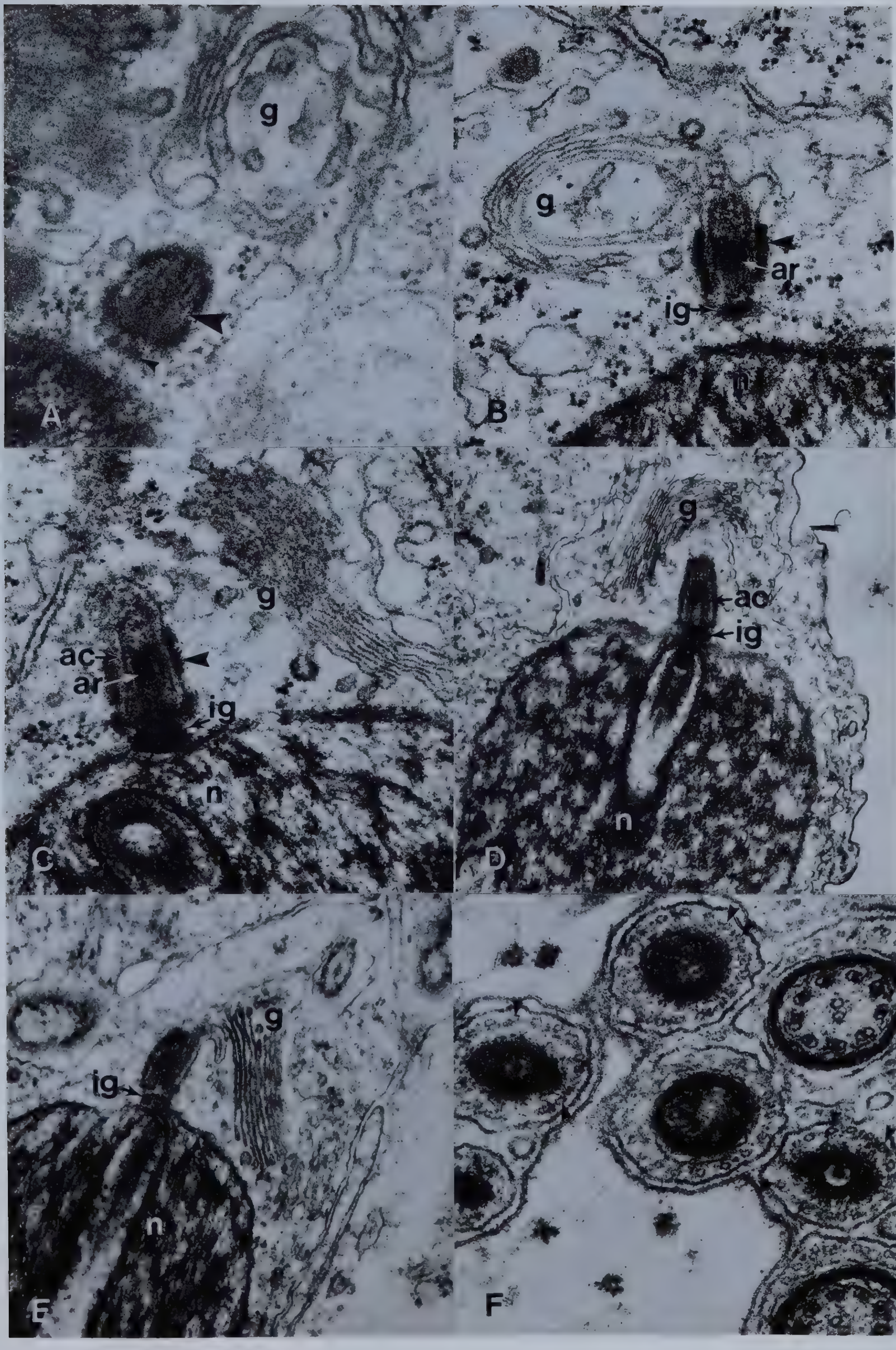

g
A
g
ar
ig
n
B
g
ac
ar
ig
n
C
g
ac
ig
n
D
g
ig
n
E
F

PLATE 13

Figure A. T-S through stage B spermatid, showing evenly dispersed, granular chromatin, x28,000.

Figure B. T-S through stage C spermatid, showing formation of fibrous strands of chromatin in nucleus. Note: Condensation of chromatin at periphery and centre of nucleus (arrows), x30,000.

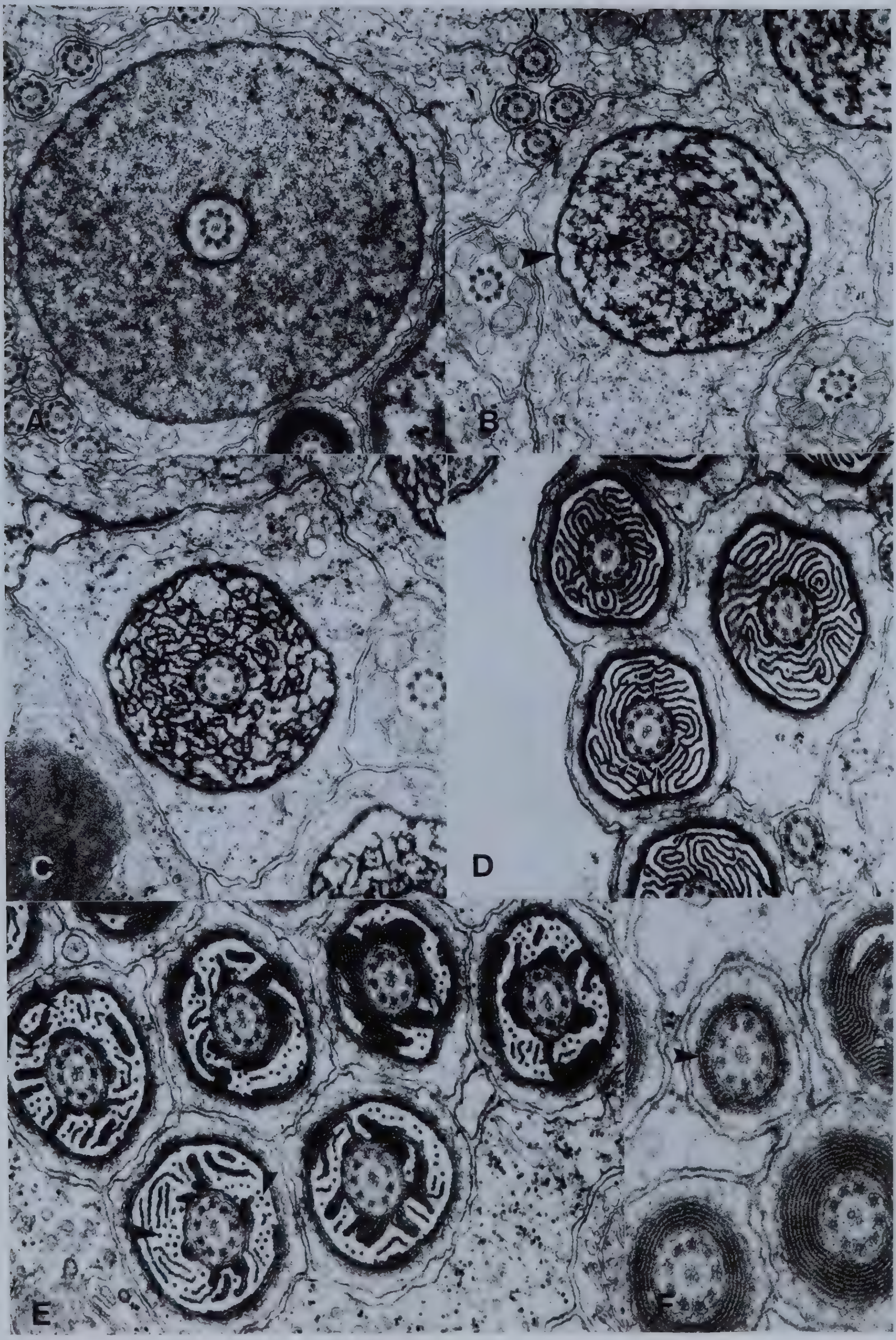
Figure C. T-S through stage C spermatid, showing preliminary stage in lamellar plate formation, x35,000.

Figure D. T-S through stage D spermatid, showing tubule arrangement adjacent to inner nuclear membrane (arrows). Distinct lamellar plates have begun to condense concentrically at periphery of nucleus. Fibres of chromatin not visible in this section, x37,000.

Figure E. T-S through stage D spermatid, showing individual fibres between lamellar plates (arrows). Condensation of plates has begun both at periphery and at centre, x45,000.

Figure F. T-S through late spermatid showing homogeneously-packed, concentric lamellae. Note: radial arrangement of chromatin persists at anterior end of nuclear tube (arrow), x50,000.

All micrographs made from sections of mature testis of *L.sitkana*.


A
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F

PLATE 14

Figure A. L-S through late spermatid, showing homogeneously dense nuclear tube anteriorly (top right), with incomplete condensation posteriorly (lower left), x13,000.

Figure B. T-S through posterior portion of late spermatid, showing irregular folding during condensation of lamellar plates. Note: fibres appear circular in cross section (arrows), x40,000.

Figure C. L-S through anterior portion of late spermatid, showing a final stage in condensation, to form homogeneous, concentric whorls of lamellae (arrows). A few fibres still persist, x40,000.

Figure D. Longitudinal grazing section through anterior portion of nucleus of stage D spermatid, showing the spiral twisting of lamellar plates (arrow), x13,000.

Figure E. T-S through late spermatids showing unilateral arrangement of microtubules beside homogeneously-dense nuclear tube (arrow). This also is visible in other sections in this micrograph, x40,000.

All micrographs made from sections of mature testis of *L. sitkana*.

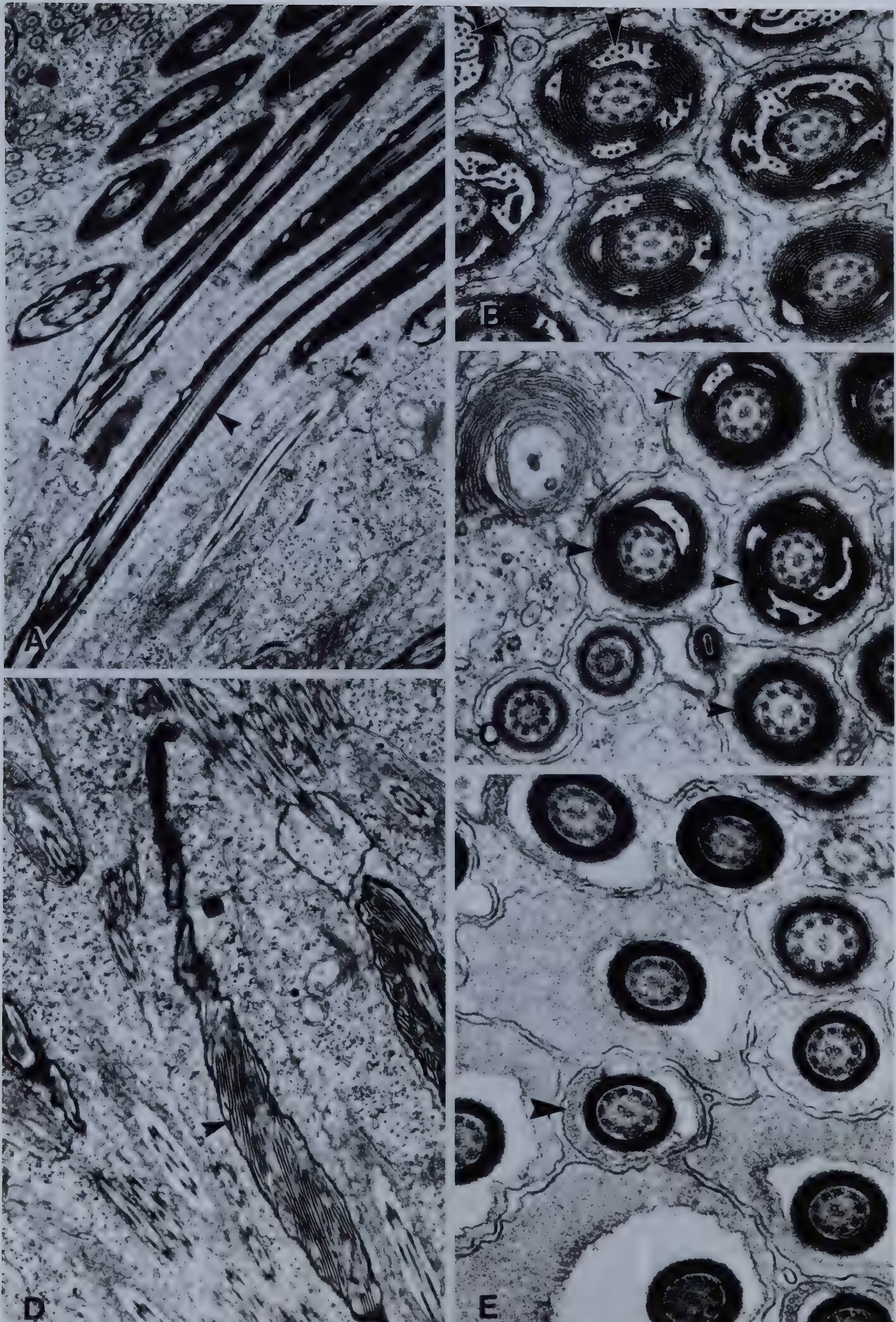
A
B
D
E

PLATE 15

Photomontage of testicular wall of *L. sitkana*, showing cytoplasm of nurse cell extending in a pseudopodium (arrow) between developing stage C spermatids. Within nurse cell cytoplasm are several lipid droplets (li) and lysosomes containing lipid droplets in the process of formation (ly). Mitochondria and dense granules are also visible, x12,500.

156

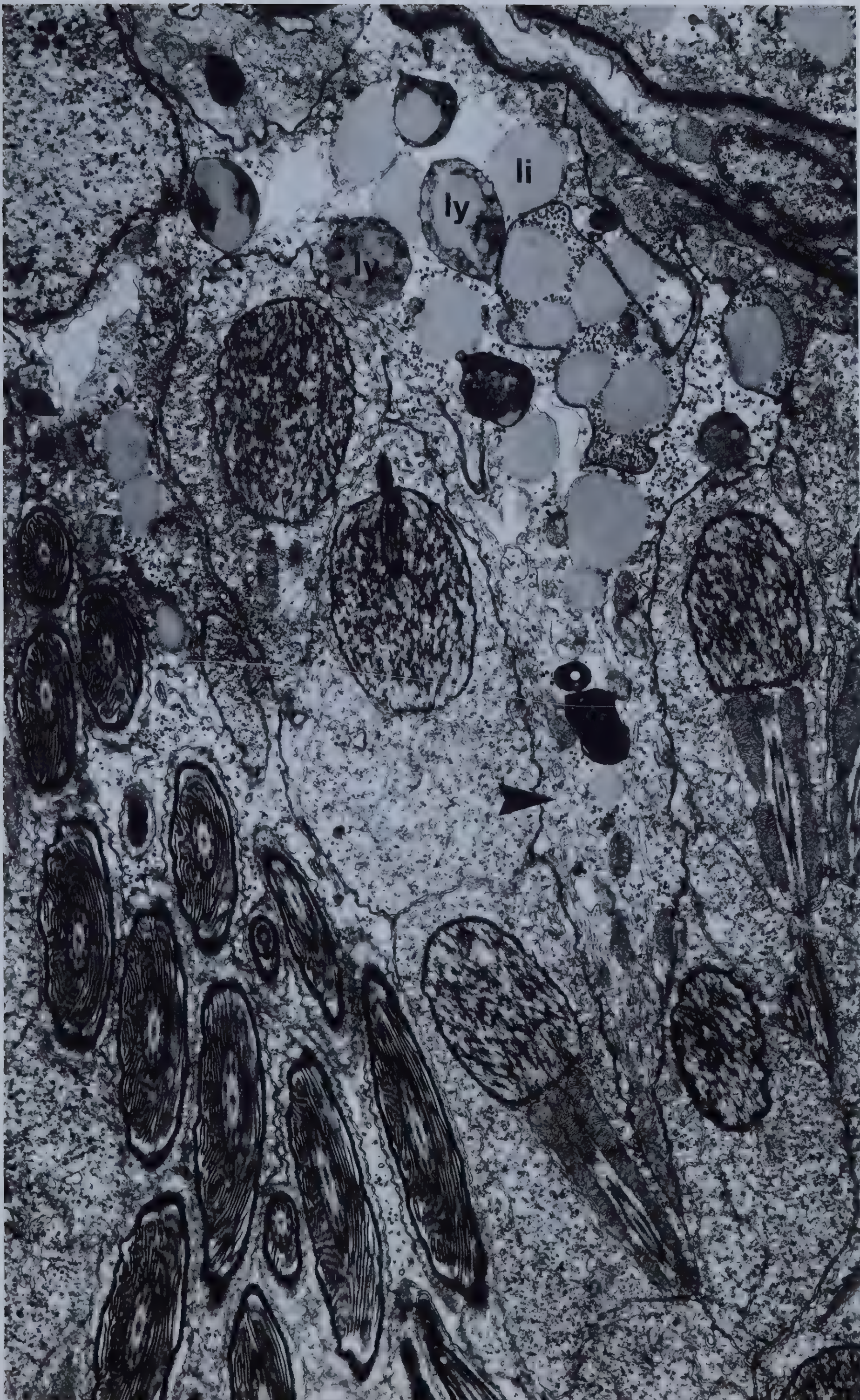

PLATE 16

Figure A. Three nurse cell pseudopodia interdigitate with an adjacent nurse cell (large arrows). Pseudopodia contain; mitochondria (m), lipid droplets (li), and mixed endoplasmic reticulum (small tailed arrow). Note: Lysosome-like body (ly), and glycogen rosettes (small arrows) may be seen in adjacent nurse cell cytoplasm, x26,500.

Figure B. Micrograph of nurse cell pseudopodium from Figure A at higher magnification. Note: double membrane structure (arrows), that results from interdigitation of plasma membranes of adjacent nurse cells, x55,000. Inset: desmosome-like junction between plasma membranes of nurse cell (nc) and spermatid (sp), x65,000.

Figure C. Cytoplasm of nurse cell (nc) adjacent to several spermatids (sp). Note: myeloid figures within lysosome (arrow); lipid droplets and glycogen rosettes are also visible, x13,000.

Figure D. Low power electron micrograph of nurse cell (nc), with attached sperm clump (sp), x5,000.

Micrographs A-C, made from sections of mature testis of *L.sitkana*. Micrograph D made from same tissue of *L.scutulata*
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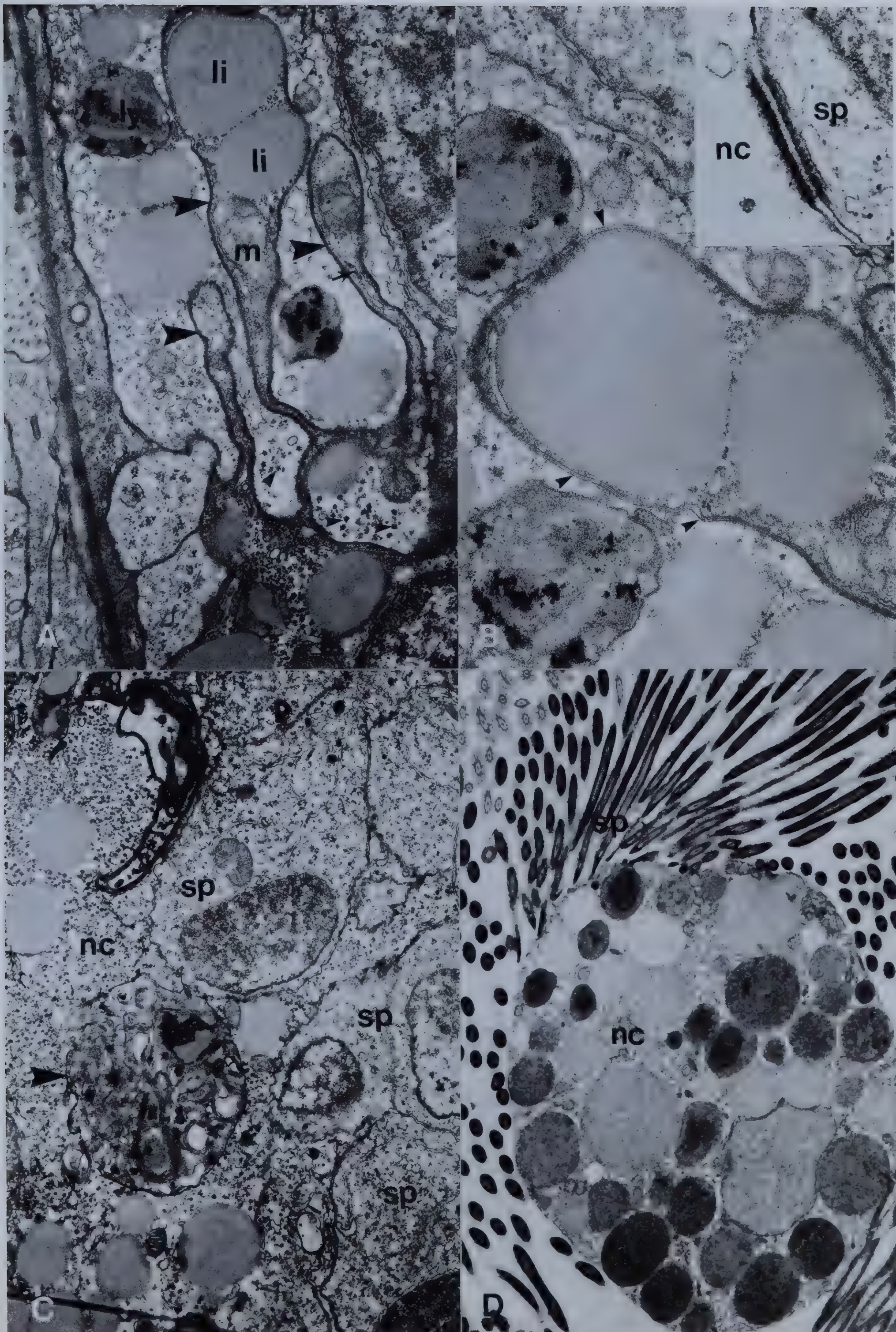
li
ly
li
m
A
sp
nc
B
sp
nc
sp
sp
C
sp
nc
D

PLATE 17

Figure A. Nurse cell in testicular tubule, surrounded by developing sperm. The nucleus (n) has a basal indentation (arrow), filled with pale-staining vesicles. Secretion droplets, in cytoplasm, are undergoing production in granular endoplasmic reticulum. There are many mitochondria, of which one is indicated (m), x17,500.

Figure B. Nurse cell in testicular tubule. Nucleus has undergone some degeneration, secretion droplets (s) have increased in size, and membrane whorls (mw), which resemble myeloid figures, have appeared in cytoplasm. A golgi body (g) and many mitochondria are also visible, x15,000.

All micrographs made from sections of mature testis of *L.sitkana*.

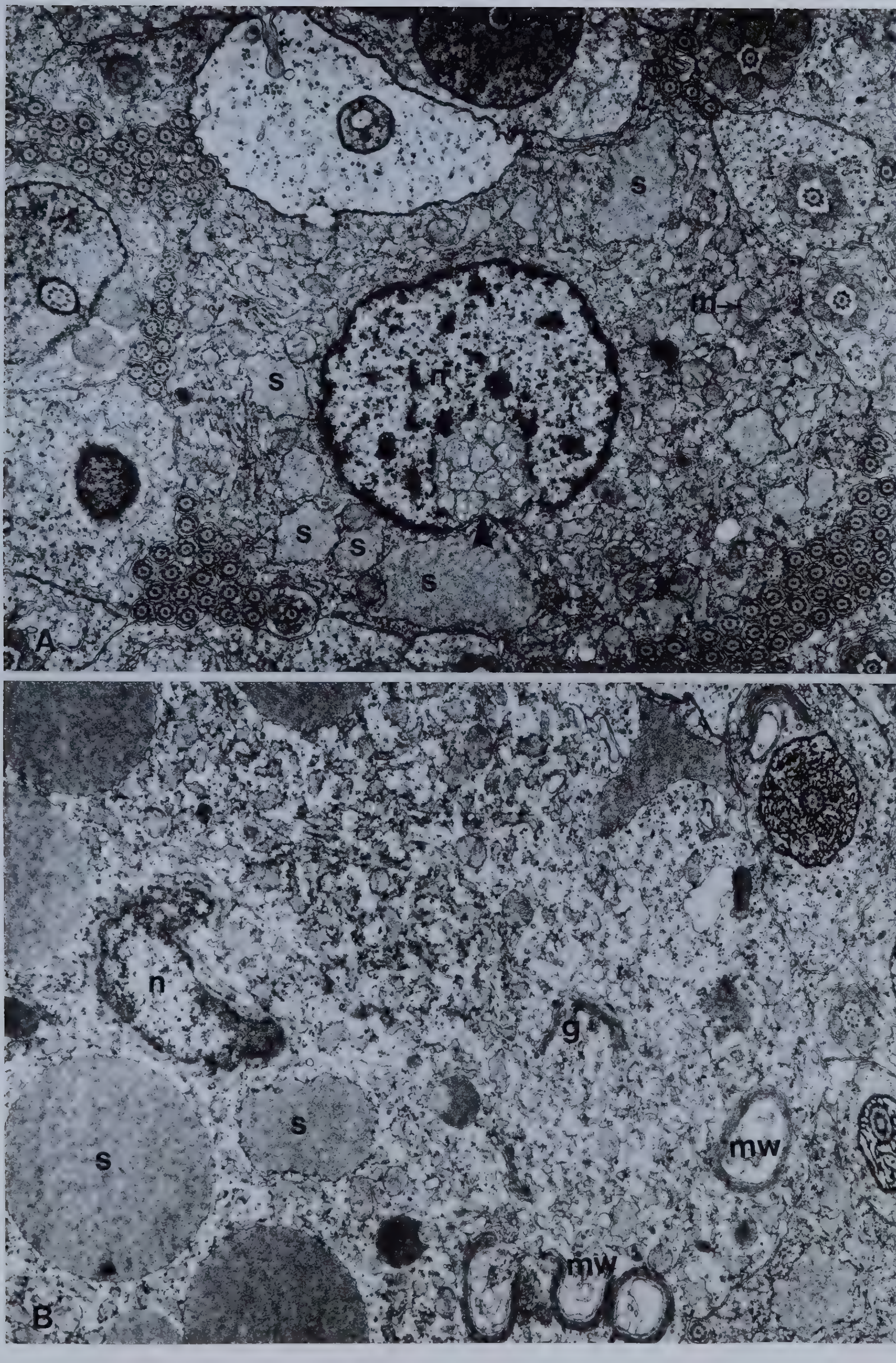
s
m
n
s
s
s
s
A
n
g
s
s
mw
mw
B

PLATE 18

Figure A. Nearly mature nurse cell surrounded by stage D spermatids (sp). Nucleus has degenerated. Large secretion droplets (s) within granular endoplasmic reticulum and a few mitochondria (m) are visible in cytoplasm, x35,000.

Figure B. L-S through acrosome, showing indistinct blebs at anterior tip (arrows), x126,000.

Figure C. Junction between plasma membranes of spermatid (sp) {acrosome region} and nurse cell (nc), showing septae that bridge them (arrows), x126,000.

All micrographs made from sections of mature testis of *L.sitkana*.

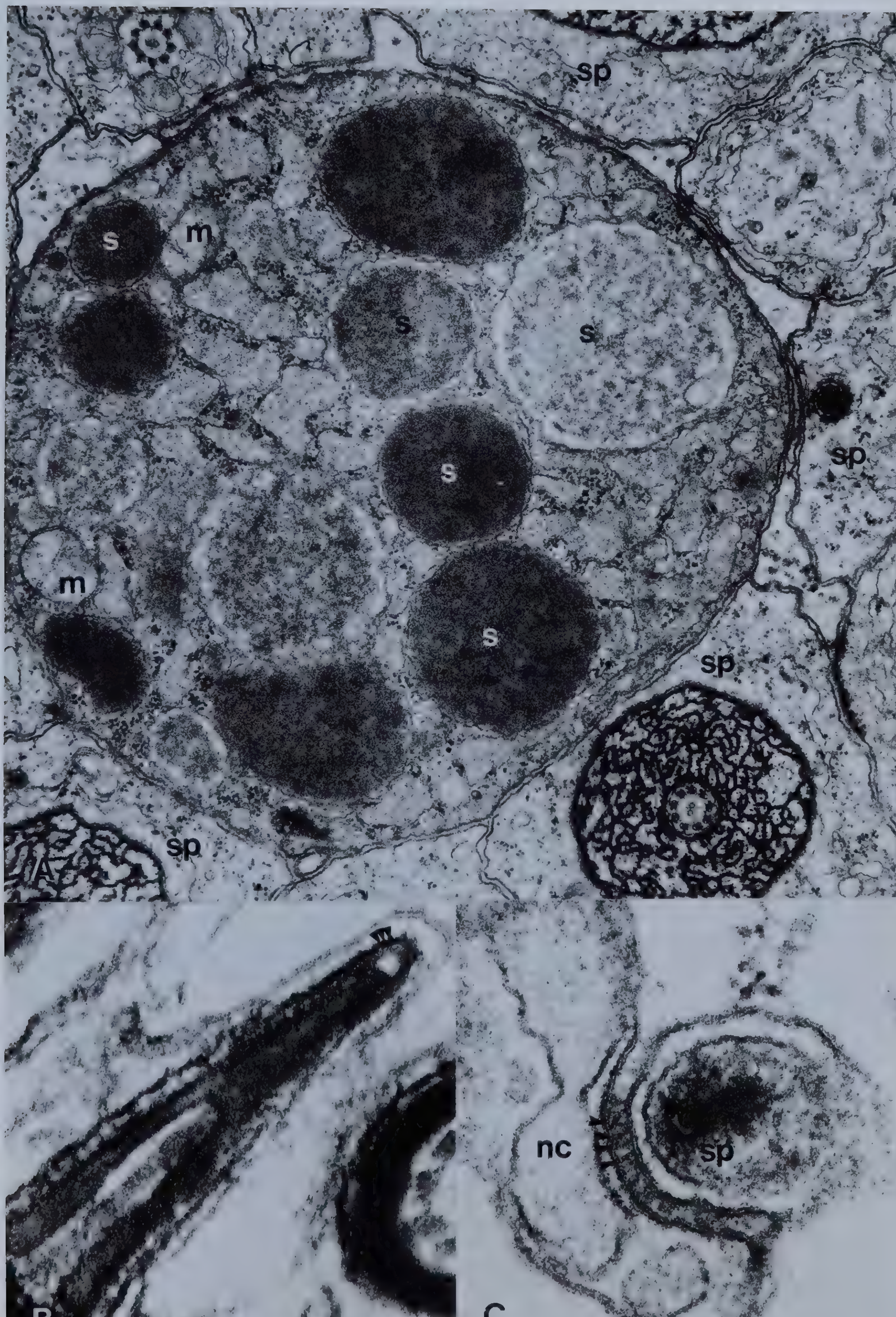
sp
s
m
s
s
s
sp
m
s
sp
sp
A
B
nc
sp
C

PLATE 19

Diagrammatic Representation of the Major Events in Spermiogenesis

Figure A. Stage A spermatid showing formation of thickened region at base (differentiated zone) and apex of nucleus (N). The distal centriole has attached to the plasma membrane by the ring centriole (arrow). A golgi body (G), mitochondria (M), two dense bodies, and scattered mixed endoplasmic reticulum are also visible. x30,000.

Figure B. Stage B spermatid showing nucleus in 'donut' stage. Centrioles have migrated to base of differentiated zone taking plasma membrane with them as a cylindrical vesicle. The distal centriole has begun producing the flagellum (F). Near the golgi body (G), is the presumptive interstitial granule (Large arrow) and acrosomal granule (small arrow). Mitochondria and scattered mixed endoplasmic reticulum are also visible, x35,000.

Figure C. Stage C spermatid. Acrosome formation has begun. Note: Golgi body (G), acrosome (A), interstitial granule (IG), golgi vesicles (v). The centriolar complex consisting of basal body (BB) and distal centriole (DC) has reached a position in the apex of the nucleus. The flagellum (f) passes back along the intranuclear canal and between the giant Nebenkerne (m) in the mitochondrial region. The tail region is not shown except for the ring centriole which appears as two dense bands (arrows) at the mitochondrial-tail junction. Chromatin condensation has begun in the nucleus (N). Dense bodies and mixed endoplasmic reticulum may also be seen, x40,000.

Figure D. Stage D spermatid. The completed acrosome is atop the nucleus. The golgi body has migrated posteriorly and can be seen lateral to the nucleus. Chromatin condensation in the nucleus has taken the form of lamellar plates which have begun to twist in a spiral. The Nebenkerne have become much elongated and twisted around the flagellar axis. The ring centriole, which is located in the joint, at the mitochondrial-tail junction, is visible at the bottom of the diagram. Reduced endoplasmic reticulum is visible in the cytoplasm, x30,000.

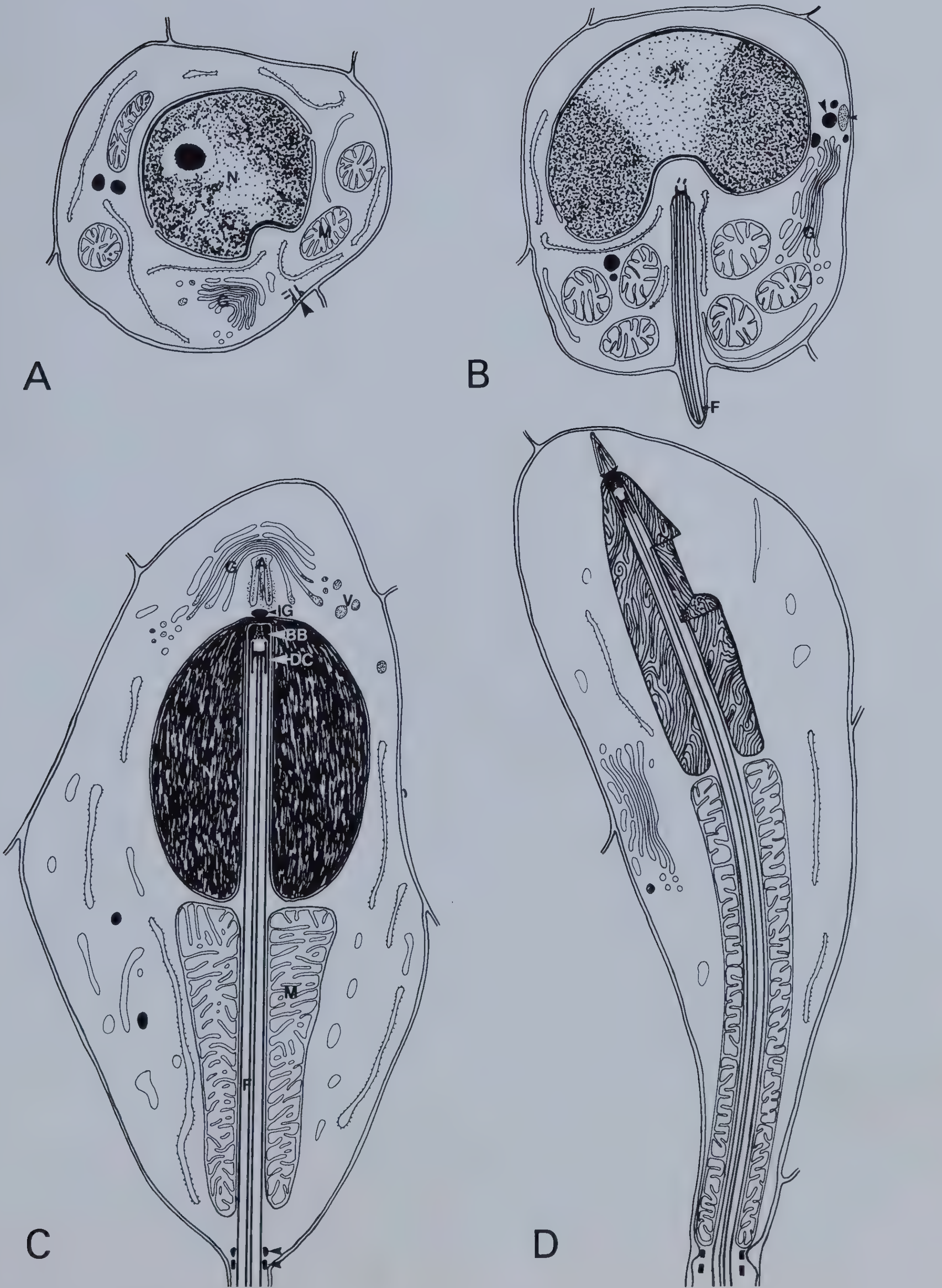
N
M
G
A
G
F
B
G
IG
BB
DC
V
M
F
C
D

PLATE 20

Figure A. L-S through acrosomal region of mature sperm. Note: acrosomal membrane (am), acrosomal granule (ac), interstitial granule (ig), invagination of nuclear envelope (i), plasma membrane of sperm (pm), acrosomal rod (ar), fine tubules (ft), x135,000.

Figure B. T-S through apical third of nuclear region showing membranous vesicle overlying flagellar fibres 7, 8 and 9 (white arrows), nucleus (n), and nuclear envelope (ne), x100,000.

Figure C. L-S through apical third of nuclear region, showing termination of nuclear envelope (large arrows) and membranous vesicle outside nucleus (small arrow), x50,000.

All micrographs made from sections of ripe seminal vesicles of *L. scutulata*.

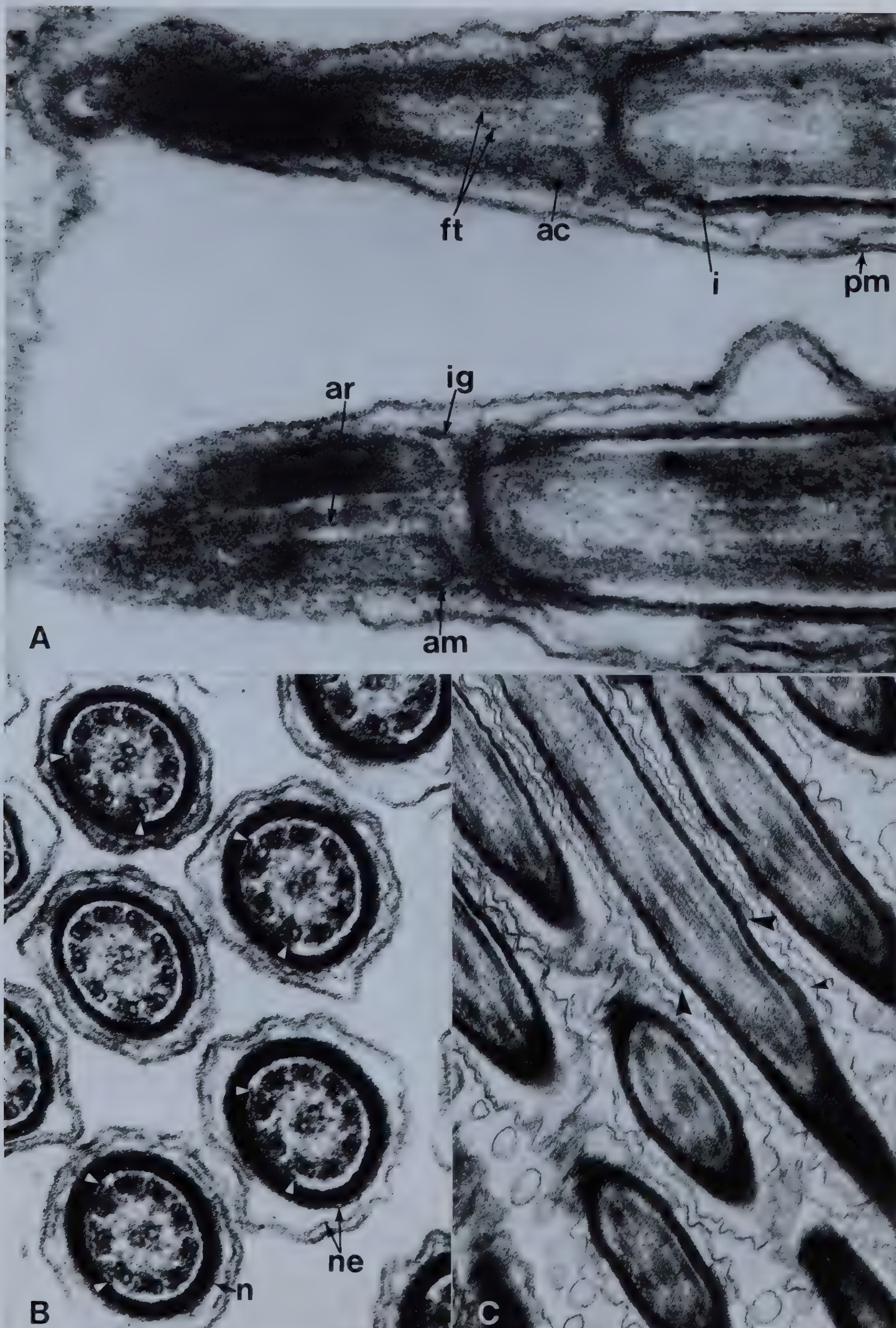
ft
ac
i
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PLATE 21

Figure A. T-S through apical third of nuclear region. Note: a, section behind distal centriole; b, section just distal to basal body; c, section through distal centriole. White arrows indicate single microtubule adjacent to internal surface of nucleus, between flagellar fibres 5 and 6, x90,000.

Figure B. T-S through basal third of nuclear region. Note: Single microtubule adjacent to external surface of nucleus between flagellar fibres 3 and 4, x43,000.

Figure C. T-S through basal body. Peripheral fibres appear to be formed from single connecting strands (black arrows). Note: invagination of nuclear envelope (white arrow), x145,000.

Figure D. Same as Figure C, x145,000.

Figure E. T-S through tip of nucleus. Note: a, section of basal plate; b, section of basal body, x100,000.

Figure F. T-S through distal centriole showing, pattern of fibrous connections between peripheral fibres, x133,500.

Figure G. T-S through distal centriole showing single connecting strand linking peripheral fibres (arrow), x110,000.

Figure H. T-S just behind basal body showing single connecting strand linking peripheral fibres (arrow), x130,000.

Figure I. L-S through apical third of spermatozoan. Note: basal body (bb); peripheral fibres (pf); central fibres (cf); origin of central fibres in distal centriole (arrow), x85,000.

All micrographs made from sections of ripe seminal vesicles of *L.scutulata*.


a
b
c
A
B
C
D
a
b
E
F
bb
pf
cf
G
H
I

PLATE 22

<u>Figure</u> <u>A</u>. T-S just behind distal centriole showing hook formed by outer arm of subfibre A of each peripheral doublet (white arrows), and connection of microtubules between doublets 5 and 6 with the outer arm of doublet 5 (black arrows), <u>x110,000</u>.

<u>Figure</u> <u>B</u>. L-S through junction of mitochondrial and nuclear regions. Note: nucleus (n), mitochondrial Nebenkerne (m), central fibres (cf), peripheral fibres (pf), mitochondrial sheath (ms), plasma membrane of sperm (pm). Arrows indicate pores in mitochondrial region, <u>x53,000</u>.

Both micrographs made from sections of ripe seminal vesicles of <u>L.scutulata</u>.

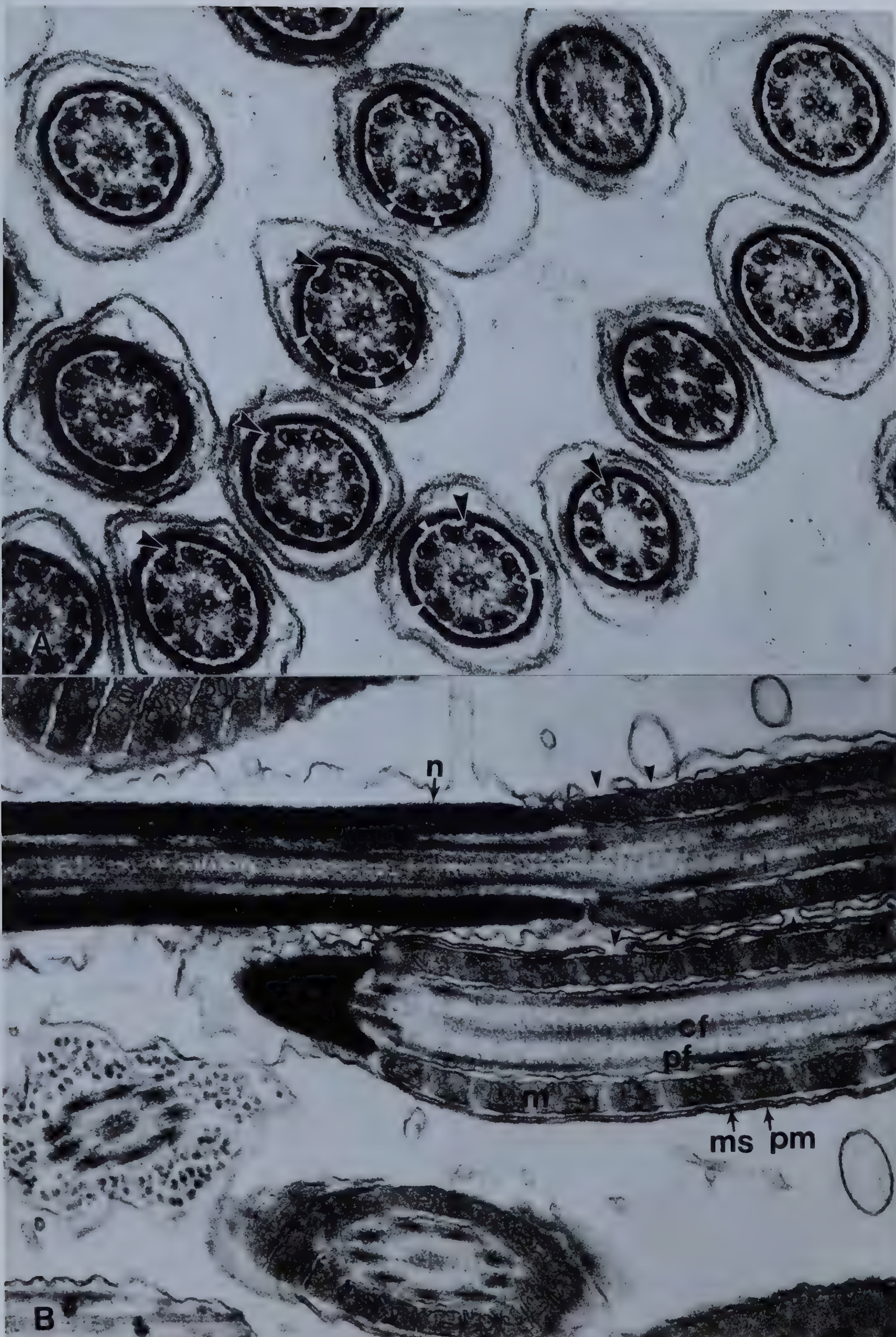

A
B
n
cf
pf
m
ms
pm

PLATE 23

Figure A. Grazing section through stage D spermatid showing, spiral arrangement of chromatin lamellae, x25,000.

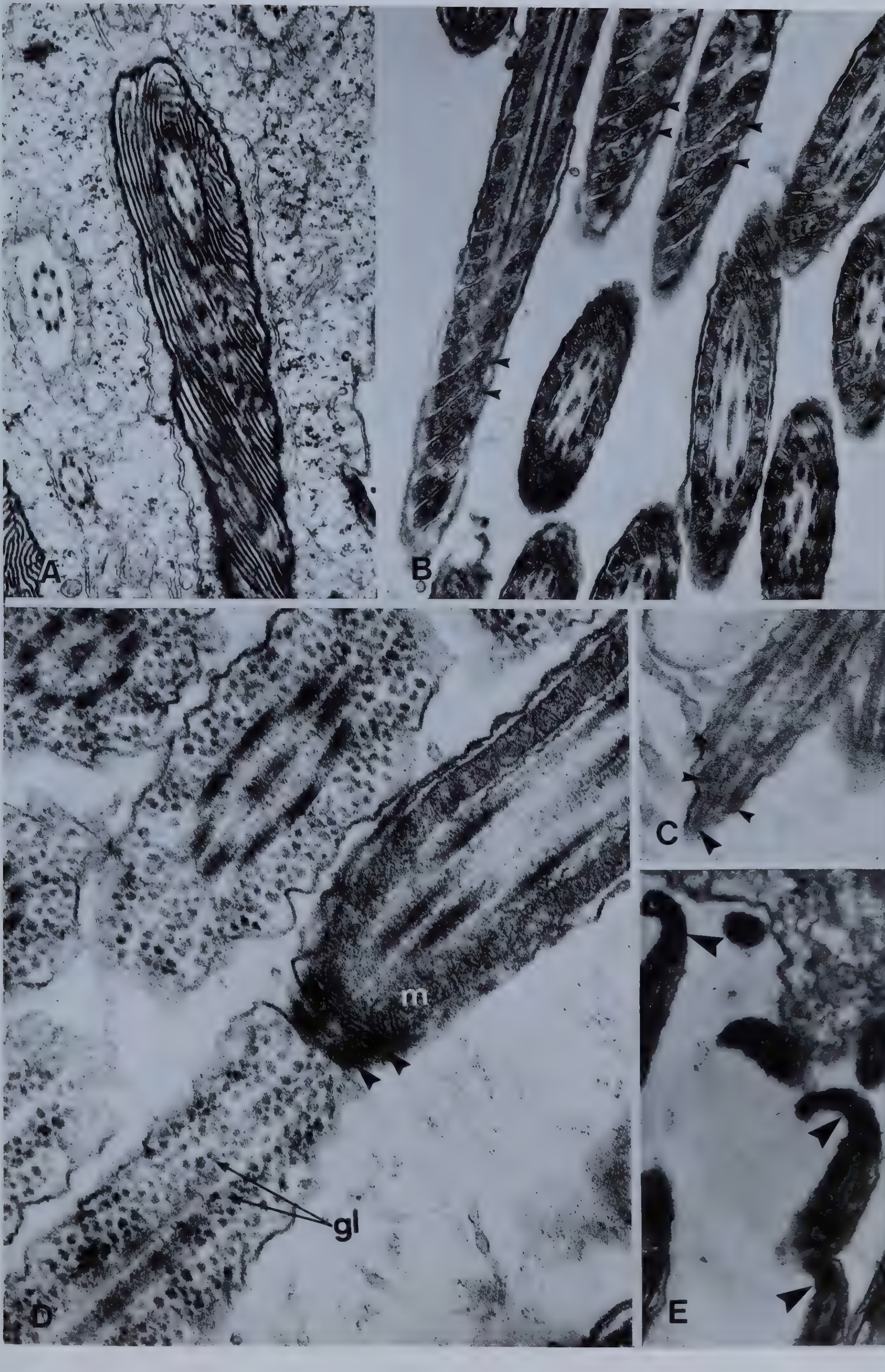
Figure B. Longitudinal grazing sections through mitochondrial region showing, spiralled Nebenkerne (arrows), x30,000.

Figure C. L-S at end of tail showing termination of peripheral fibres (small arrows) and central fibres (large arrows), x61,000

Figure D. L-S through junction of mitochondrial and tail regions. Note: ring centriole (arrows); glycogen particles (g); mitochondrial Nebenkerne (m), x55,000.

Figure E. L-S through acrosome illustrating its flexibility (arrows), x40,000.

All micrographs made from sections of ripe seminal vesicles of *L. scutulata*.


A
B
C
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m
gl

PLATE 24

Figure A. Scanning electron micrograph at junction between tail and mitochondrial regions. Note: joint (arrows), x5,000.

Figure B. L-S of junction between tail and mitochondrial regions showing, joint (arrow) and ring centriole (rc). Note: Spiralling of peripheral fibres (pf), x35,000.

Figure C. Scanning electron micrograph of joint between tail and mitochondrial regions showing how tail can beat perpendicularly to main axis of sperm. Note: joint (arrow) and spiralled Nebenkerne in mitochondrial region (m), x5,000.

Figure D. L-S through tail region during perpendicular phase of beat. Note: tail (t), mitochondrial region (m), flagellar shaft (f) seen in cross section here, but in long section in tail region, x26,000.

All micrographs made from preparations of ripe seminal vesicle tissue of *L. scutulata*.

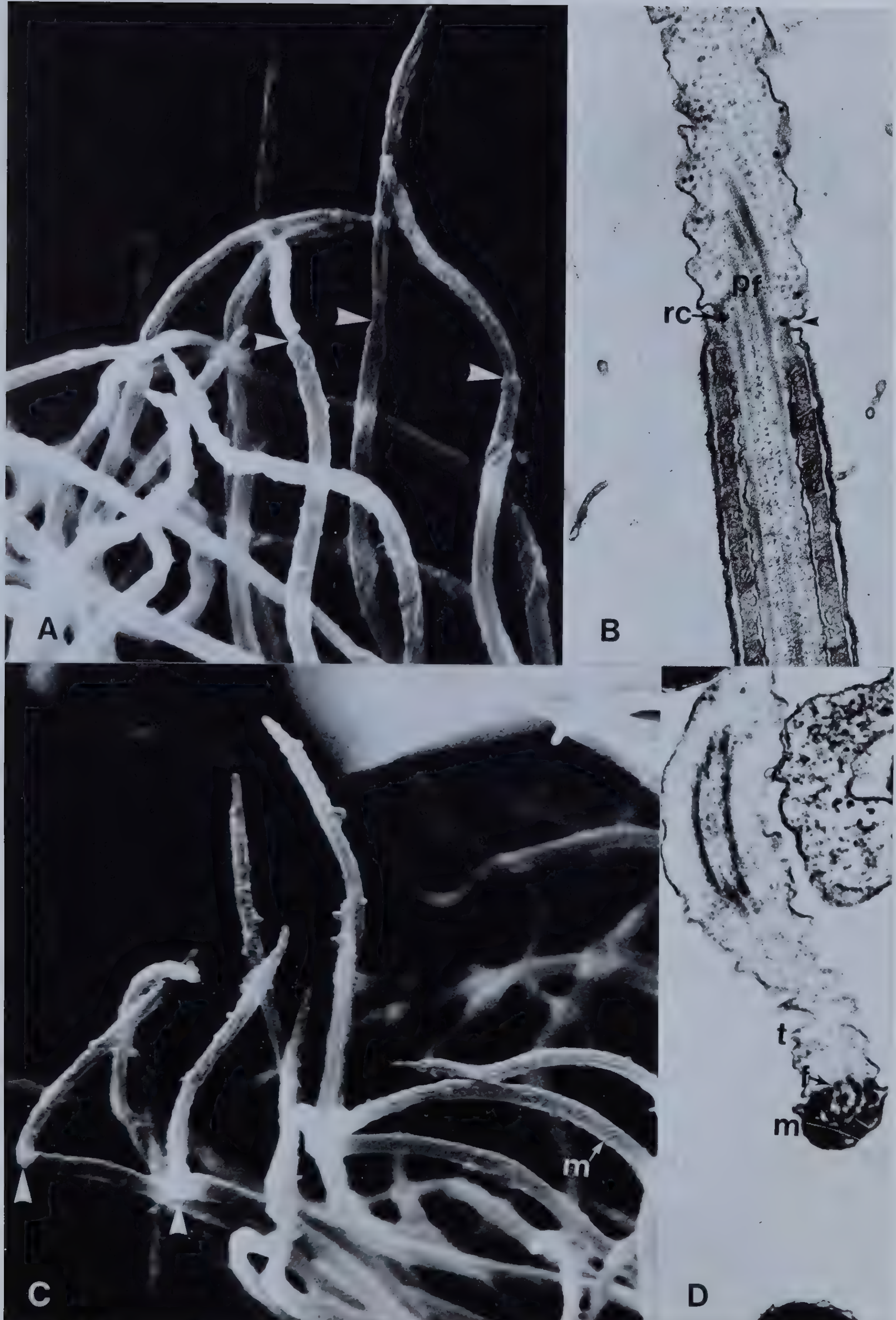
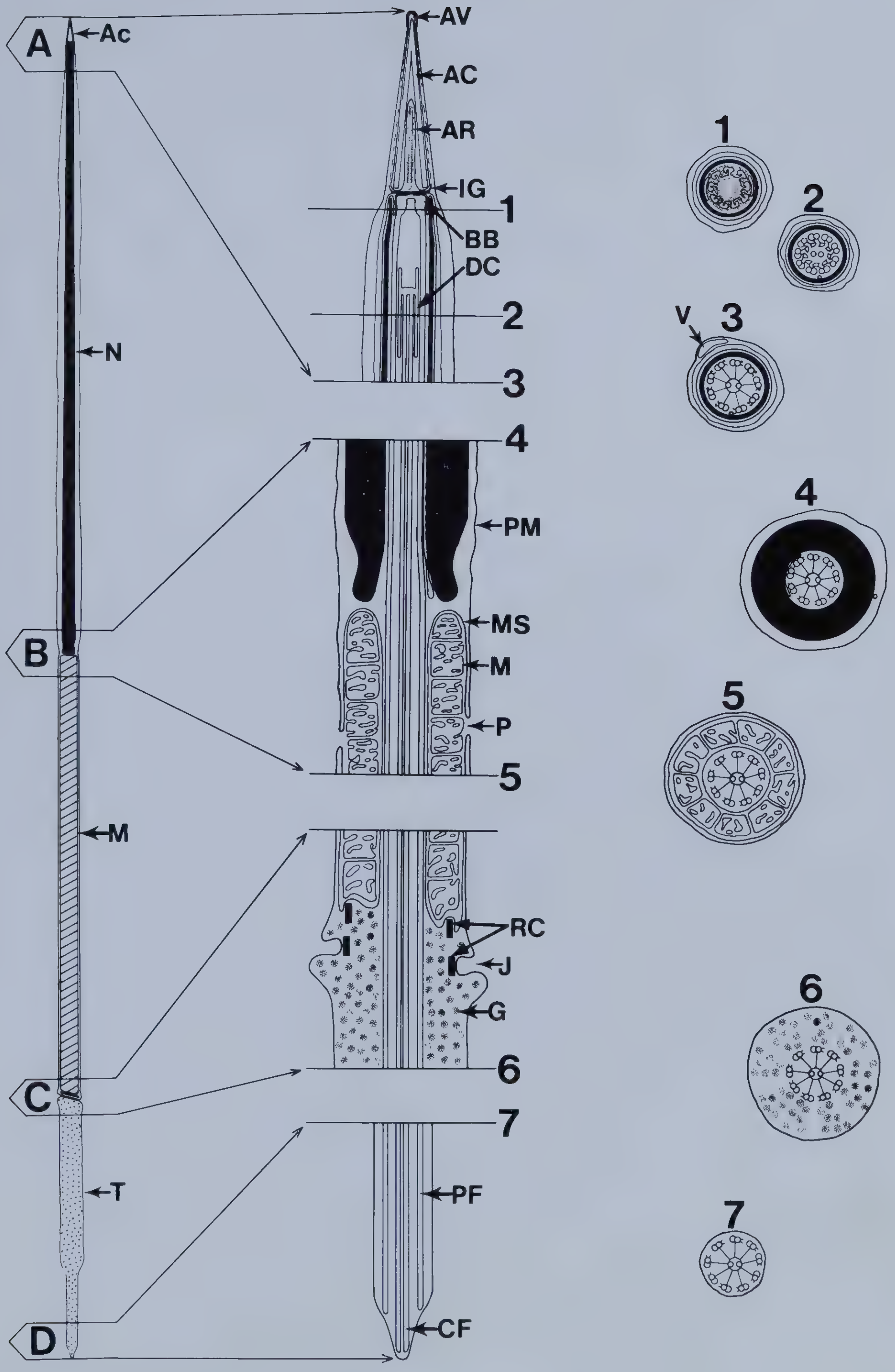

A
B
rc
pf
C
m
D
t
f
m

PLATE 25

Diagrammatic representation of the composite ultrastructural features of the spermatozoon of *Littorina scutulata* : LEFT ; general distribution diagram of whole sperm, indicating the four main regions. Acrosomal (Ac), Nuclear (N), Mitochondrial (M), Tail (T): CENTRE, longitudinal sections A - D from general diagram magnified x7. {A} Section of head of sperm showing acrosome vesicle (AV), acrosome granule (AC), acrosome rod (AR) interstitial granule (IG), basal body (BB), distal centriole (DC); {B} Section of junction between nuclear and mitochondrial regions, showing, plasma membrane in nuclear region (PM), mitochondrial sheath (MS), mitochondrial Nebenkerne (M), pore (P); {C} Section of junction between tail and mitochondrial regions showing, ring centriole (RC), joint (J), glycogen granule (g); {D} Section of distal portion of tail region showing peripheral fibres (PF), central fibres (CF): RIGHT, transverse sections of sperm taken at points indicated in diagrams at CENTRE ; 1. Section of nuclear region at site of basal body. 2. section of nuclear region at site of distal centriole. 3. Section of nuclear region just posterior to distal centriole. Note, vesicle (V). 4. Section in basal third of nuclear region. 5. Section in mitochondrial region. 6. Section in proximal portion of tail region. 7. Section in distal portion of tail region.


A
Ac
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B
M
C
T
D
AV
AC
AR
IG
1
BB
DC
2
3
4
PM
MS
M
P
5
RC
J
G
6
7
PF
CF
1
2
V
3
4
5
6
7

PLATE 26

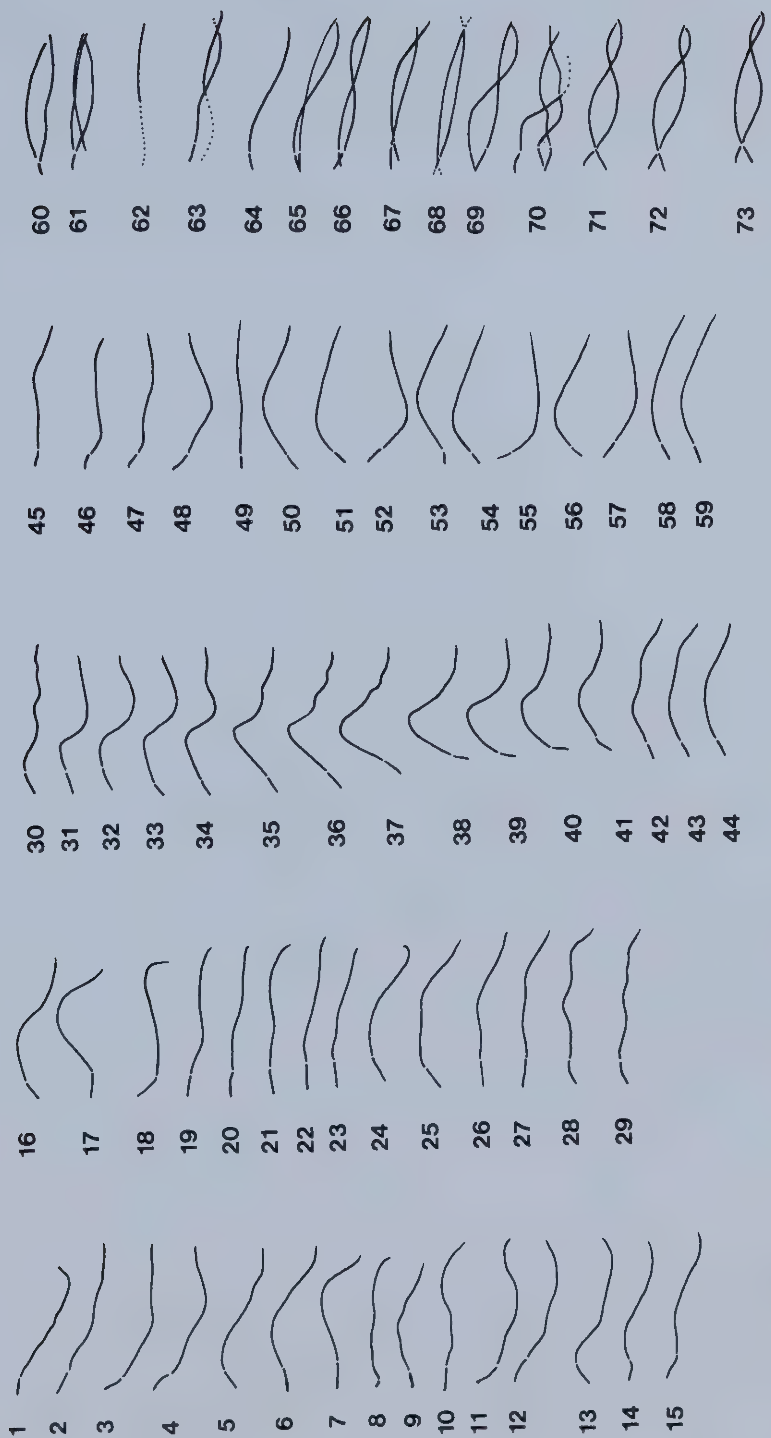
Figure A. Trinodal envelope of swimming *Littorina* sperm. Nuclear region is all black, mitochondrial region is striped, and tail region is stippled. Arrow indicates direction of movement and rotation. Node N1, is advancing at acrosome tip; node N2, is in nuclear region; node N3, is located at tail joint. Rotational frequency is 24 revs./sec. ; velocity is 185 m/sec. x900.

Figure B. Multiple flash photomicrograph of *L.sitkana* sperm, obtained with a Zeiss photomicroscope on dark field phase and a high frequency stroboscope (pulse, 50/sec.; duration, 3 secs.). Arrows indicate independent tail beat, x470.

A

PLATE 27

Tracings of a 72 frame sequence of cine film (24 frames/sec) of swimming L.sitkana spermatozoan. Frames 1 - 37 show the anti-clockwise twisting that generates super-coiling (torque) in the flagellum (frames 36, 37). Frames 38 - 57 show release of torsion and formation of binodal wave. Frames 69 - 73 show formation of trinodal wave. Note: tail joint is visible as a 'break' in the sperm at the left hand end, x500.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
16 17 18 19 20 21 22 23 24 25 26 27 28 29
30 31 32 33 34 35 36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51 52 53 54 55 56 57 58 59
60 61 62 63 64 65 66 67 68 69 70 71 72 73

PLATE 28

Figure A. An onion body (o) in cytoplasm of S-V cell is surrounded by lipoid bodies (li), mitochondria (m), and residual bodies (r), x14,000.

Figure B. Cell bud containing heterophagic vacuoles (large arrow) and enveloping sperm by membrane overgrowth (small arrow), x4,500.

Figure C. Section through part of an onion body (o), showing its close relationship with the golgi body. Large mitochondria are visible at top right, x13,500.

Figure D. Cell bud in lumen containing heterophagic vacuole (h), myelcid figures (m), telolysosome (t), residual body (r), and a lipoid body (li), together with other products of sperm digestion, x5,000.

Figure E. Cell bud containing partially digested sperm (sp) and myelcid figures (mf), in process of being resorbed by S-V cell (at bottom), x16,500.

All micrographs made from sections of seminal vesicles of *L.scutulata*.

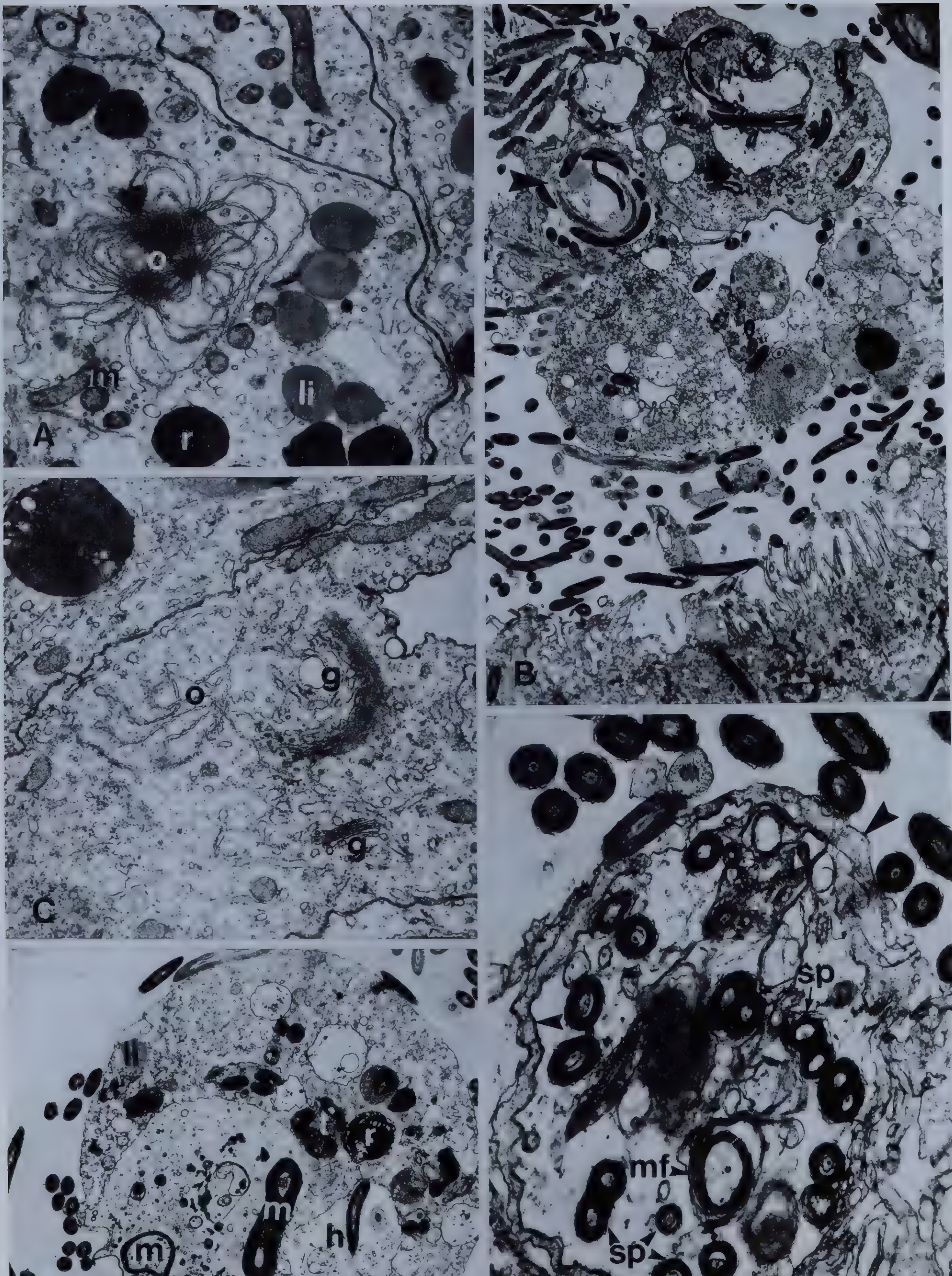

A
m
li
r
B
C
o
g
g
D
m
m
h
r
E
sp
mf
sp

PLATE 29

Figure A. Heterophagic vacuole (arrow) in S-V cell cytoplasm. Note: variety of planes of section indicating that sperm is wound up in a ball, x15,000.

Figure B. Sperm filament undergoing resorption by S-V cell (at bottom). Note: filament is not enclosed in membranes of cell bud (cf.PLATE 28E), x12,500.

Figure C. Micrograph of freeze-etched tissue taken from seminal vesicles during sperm resorption. Note: Several golgi bodies (g) and primary lysosome-like bodies (arrows), x14,500.

Figure D. Same as Figure C. Note: Folded plasma membrane (pm), x14,500.

Figure E. Multivesicular body (arrow) in process of digesting sperm (sp), x12,000.

All micrographs made from sections of seminal vesicles of *L.scutulata*.

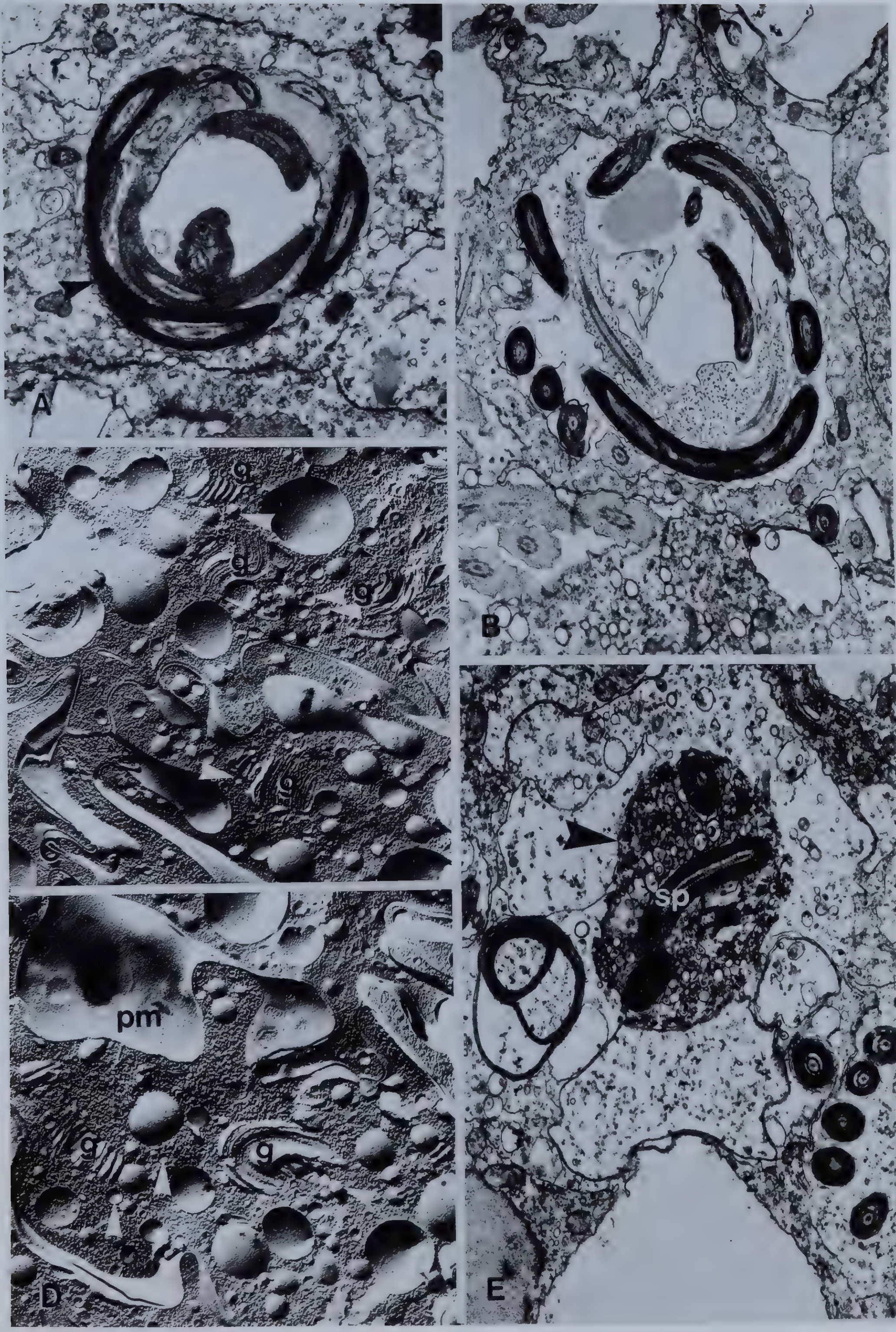

A
B
D
pm
g
g
E
sp

PLATE 30

Figure A. Myeloid figure. Close scrutiny reveals densely - packed, concentric lamellae at centre (arrow), x25,000.

Figure B. Myeloid figure, similar to that in Figure A, prepared by freeze-etching. Numerous similar structures were observed in the same area, x25,000.

Figure C. Portion of S-V cell cytoplasm, containing secondary lysosome (arrow), in conjunction with two multivesicular bodies (mb), which may be in process of fusion with the lysosome. Note: Myeloid figure (mf) at lower right, x20,000.

Figure D. Portion of S-V cell cytoplasm, containing lipoid bodies (li), residual bodies (r) - some of which are degenerating to myeloid figures (lower right), and myeloid figures (arrows), x12,000.

Figure E. Portion of S-V cell cytoplasm, containing heterophagic vacuoles (h), telolysosomes (t), and residual body (r). Note: Granular contents of residual body resemble glycogen particles in section of sperm tail (double arrow), x5,000.

Figure F. Telolyscosome in S-V cell cytoplasm, adjacent to nucleus (n), x9,000.

All micrographs made from sections of seminal vesicles of *L.scutulata*.


A
B
C
D
E
F
mb
mb
mf
li
li
li
r
r
r
r
r
h
h
h
t
n

PLATE 31

Figure A. S-V cell engulfing individual sperm filaments by membrane overgrowth (arrow). The series X - Z, demonstrates process by which sperm is engulfed in whorls of double membrane, which later degenerate, thus enclosing sperm in cell, x8,000.

Figure B. Amoebocyte engulfing waste particle (p) by membrane overgrowth (arrow), x1,700.

Figure C. S-V cells and cell buds engulfing sperm filaments by membrane overgrowth (arrows). Note: Similar sequence of events as in Figure A, x10,000.

Figure D. Low power electron micrograph of destructive S-V cell. Note: residual bodies (r); vacuole (v); lipoid bodies (li); nucleus (n), x3,000.

Figure E. S-V cell in resting phase. Note: onion body (o), residual bodies (r), lipoid bodies (li), golgi bodies (g), mitochondria (m), and uniform microvillar apex. Nucleus is absent from section. Adjacent cells are in contact via desmosome-like junctions (arrows), x11,000.

All micrographs made from sections of seminal vesicles of *L.scutulata*.

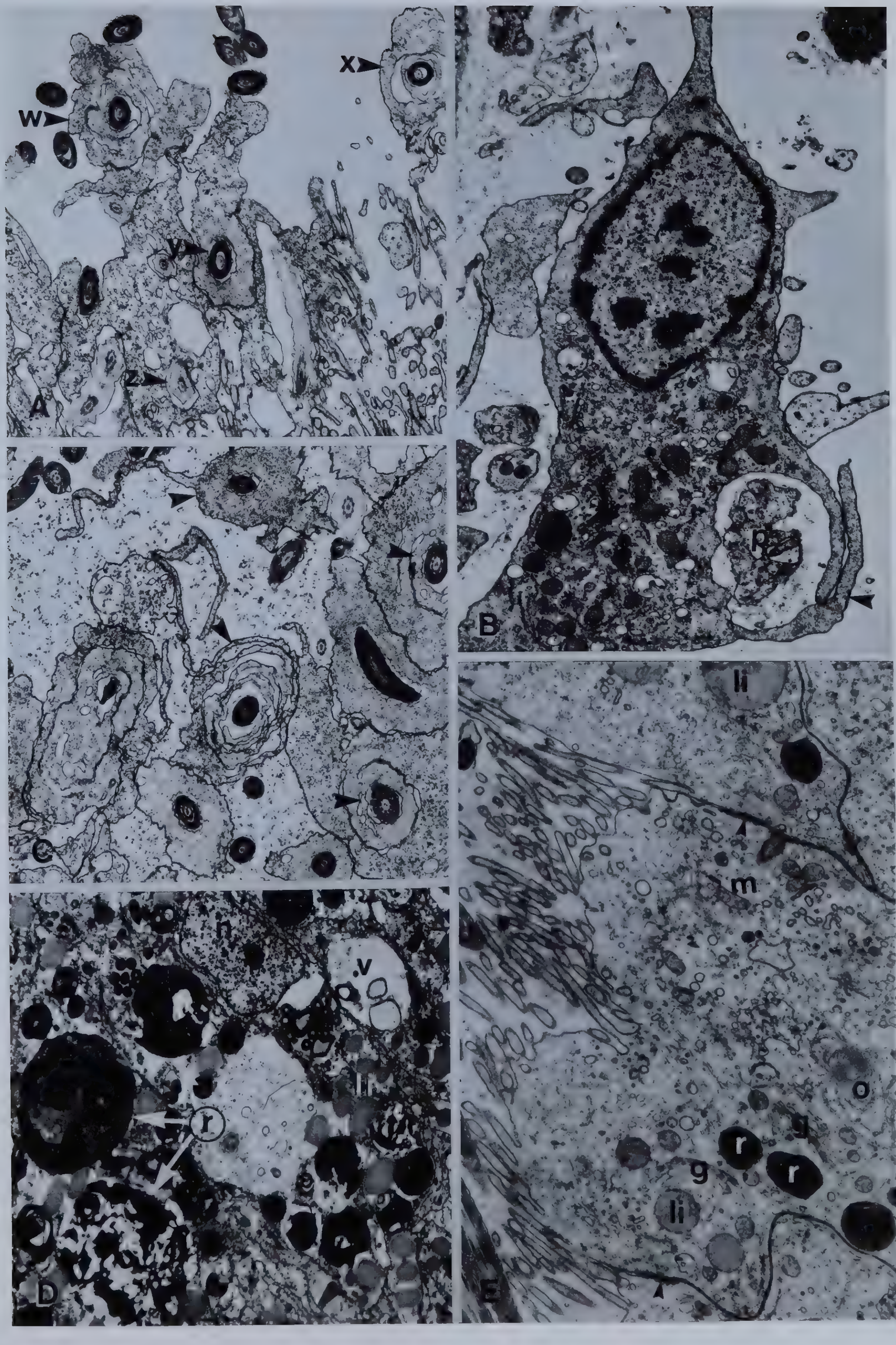

A
w
x
y
z
B
p
C
D
n
v
li
r
E
li
m
o
g
r
r
g
li

PLATE 32

Diagrammatic representation of the major events that occur during the resorption and digestion of sperm in the S-V cell. Note the following (from top to bottom): sperm filaments (sp); secondary lysosomes (SL); Vacuoles (V); Cell Buds (CB); Sperm filament in a ball (Sp); Telolysosomes (TL); Heterophagic vacuole formed from a sperm filament (HV1); Heterophagic vacuole formed from a spermatozeugma{ nurse cell+ sperm clump} (HV2); Multivesicular Body (MB); Golgi bodies (G) and Primary Lysosomes produced by them (small arrows); Residual Body (RB); Nuclei (N); Lipoid Body (LB); Lipid in Residual Body (L,RB); Myeloid Figure (My); Circular Muscle layer below basal lamina (CM); Longitudinal Muscle layer (LM); x9,000.

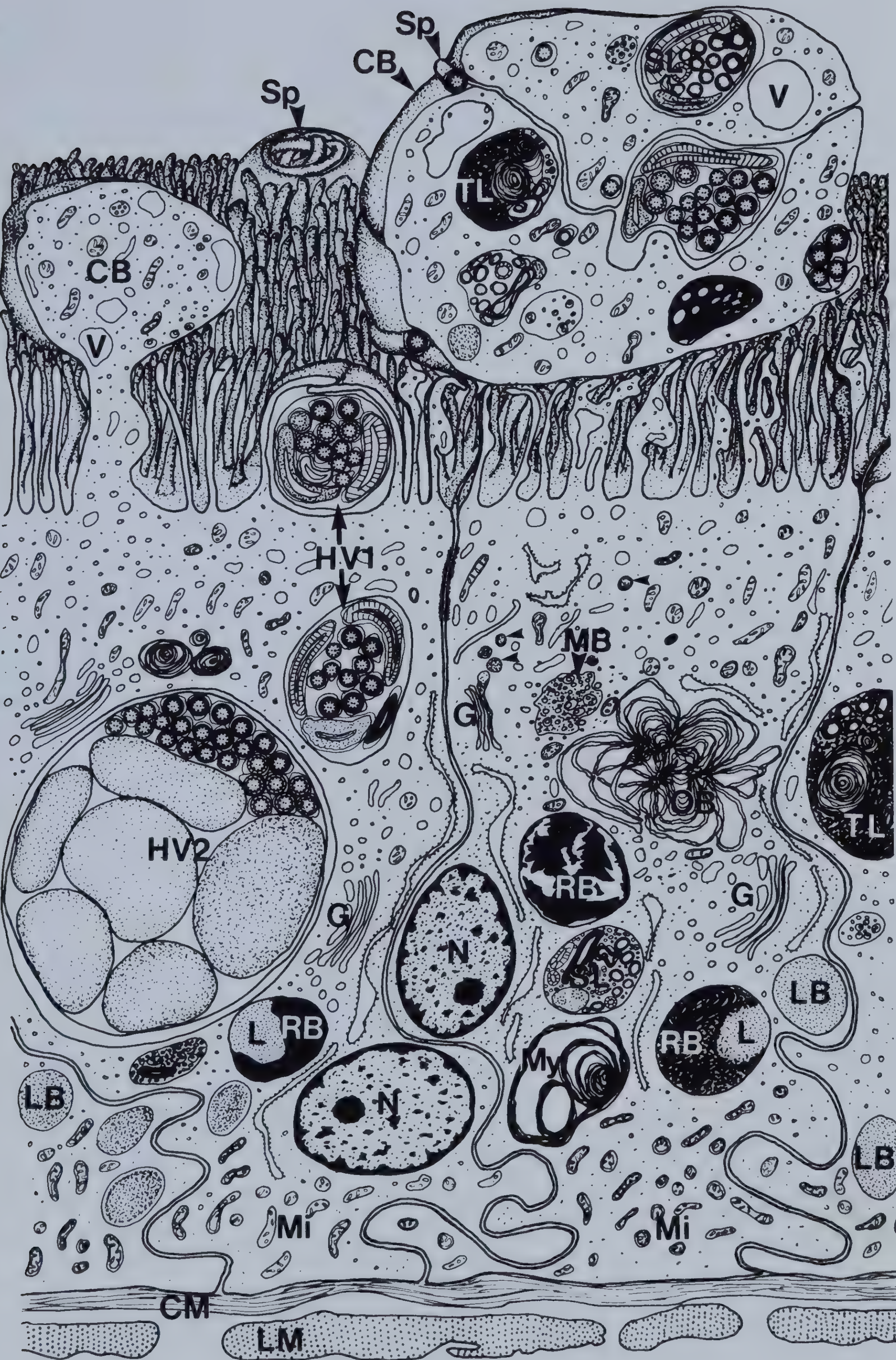

Sp
CB
Sp
SL
V
TL
CB
V
HV1
MB
G
TL
HV2
RB
G
G
N
SL
LB
L
RB
RB
L
My
LB
N
LB
Mi
Mi
CM
LM

PLATE 33

Hypothetical stages in the phagocytosis and intracellular digestion of sperm by an S-V cell (A-K) Sperm ball (A); sperm filaments (B); heterophagic vacuole (c); primary lysosomes formed from golgi body (E); early secondary lysosome (D); multivesicular body (F); late secondary lysosome (G); telolysosome (H); residual body releasing lipoid body (I); residual body containing lipid (J); lipoid body (K).

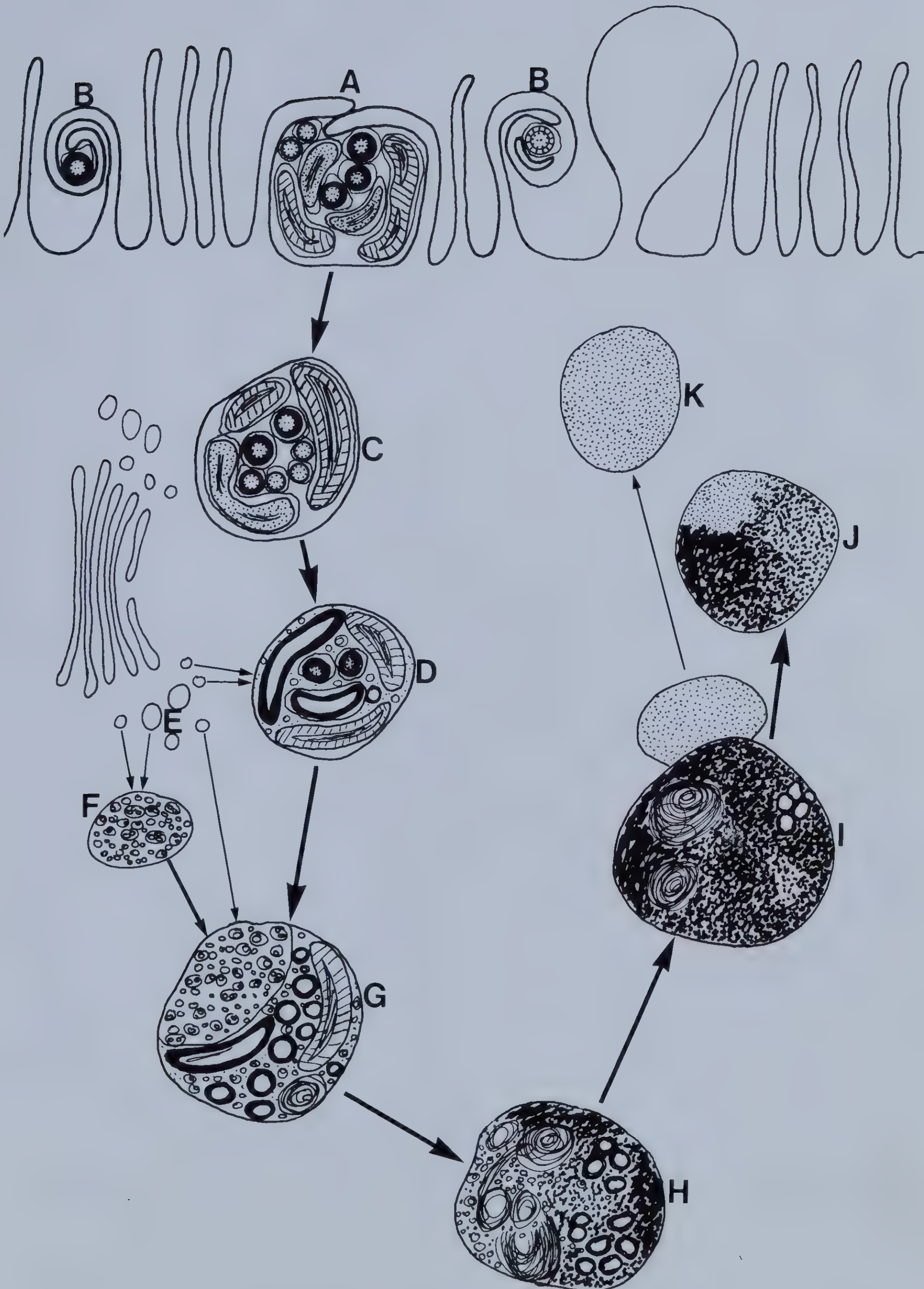

A
B
B
C
D
E
F
G
H
I
J
K

PLATE 34

Figure A. Light micrograph of sperm resorption in seminal vesicles of L.sitkana. Note: cell buds (cb) containing coiled sperm (arrows) and nurse cells (nc), x650.

Figure B. Light micrograph of sperm (sp) of L.sitkana oriented with bursa wall. Note: degenerating nurse cells and prostatic secretion in lumen, x275.

Figure C. Section through egg capsule in vestibule of L.scutulata showing the capsule fibres (arrows), rendered yellow by AB-AY, x240.

Figure D. T-S of L.sitkana penis showing arrangement of multicellular glands (g) at base of papilla. Note: long cell processes (arrows) passing through muscle layer (m), x800.

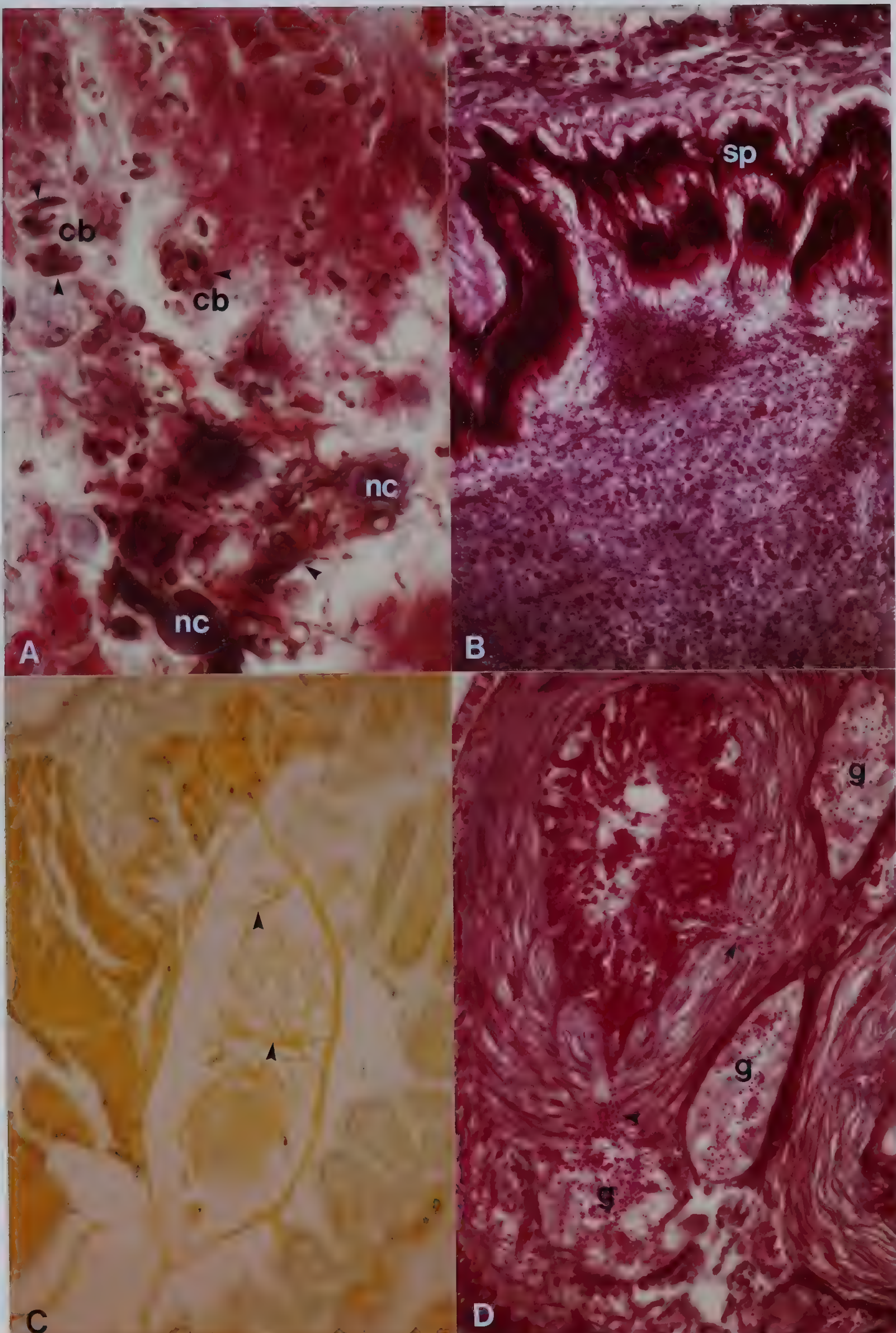
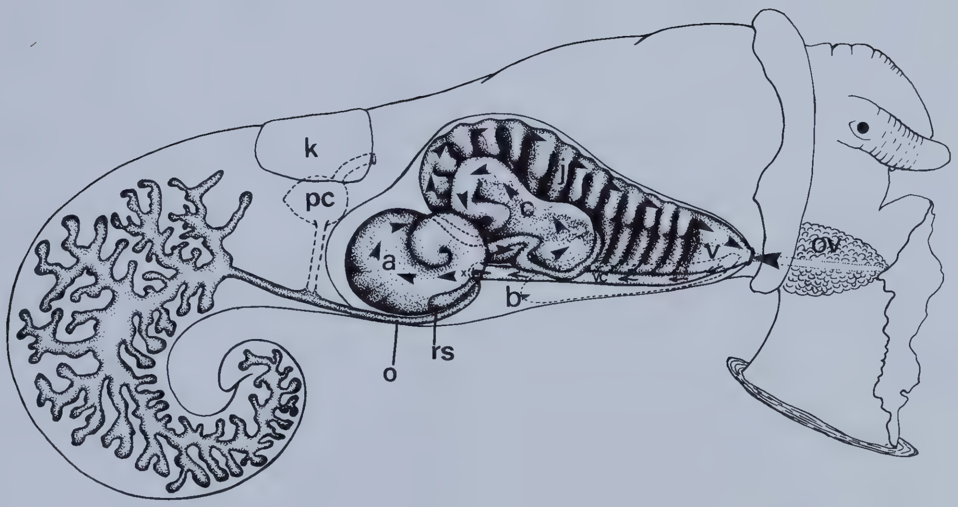

cb
cb
nc
nc
A
sp
B
C
g
g
g
D

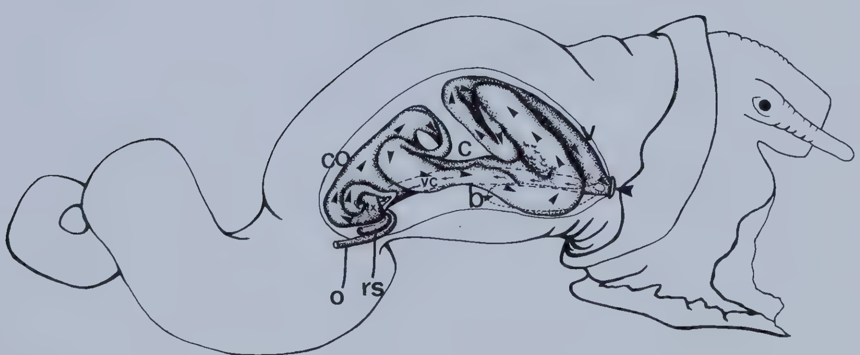
PLATE 35

Figure A. Diagrammatic representation of the ovarian tubules, renal and pallial oviduct system in L. sitkana. Ovarian tubules at left, drain into oviduct (o), leading to junction with receptaculum seminis (rs). Predicted site of fertilisation of eggs is indicated (x); Note: albumen gland (a); capsule gland (c); jelly gland (j); vestibule (v); bursa copulatrix (b); ventral sperm channel (vc); ovipositor (ov); and gonopore (largest arrow). A series of connections exists between oviduct (o), pericardium (pc), kidney (k), and mantle cavity {dotted lines}. The passage of eggs (medium arrows) and sperm (small arrows), through the system of ducts, is indicated, x9.

Figure B. Diagrammatic representation of the pallial oviduct system of L. scutulata {renal oviduct and its connections to the pericardium, etc., and ovarian tubules are similar to L. sitkana and therefore have been omitted}. Note: termination of renal oviduct (o); receptaculum seminis (rs); predicted site of fertilisation (x); covering gland (co); capsule gland (c); vestibule (v); bursa copulatrix (b); ventral channel of oviduct (vc); gonopore (largest arrow). The passage of eggs (medium arrows) and sperm (small arrows), through the system of ducts, is indicated, x9.



A



B

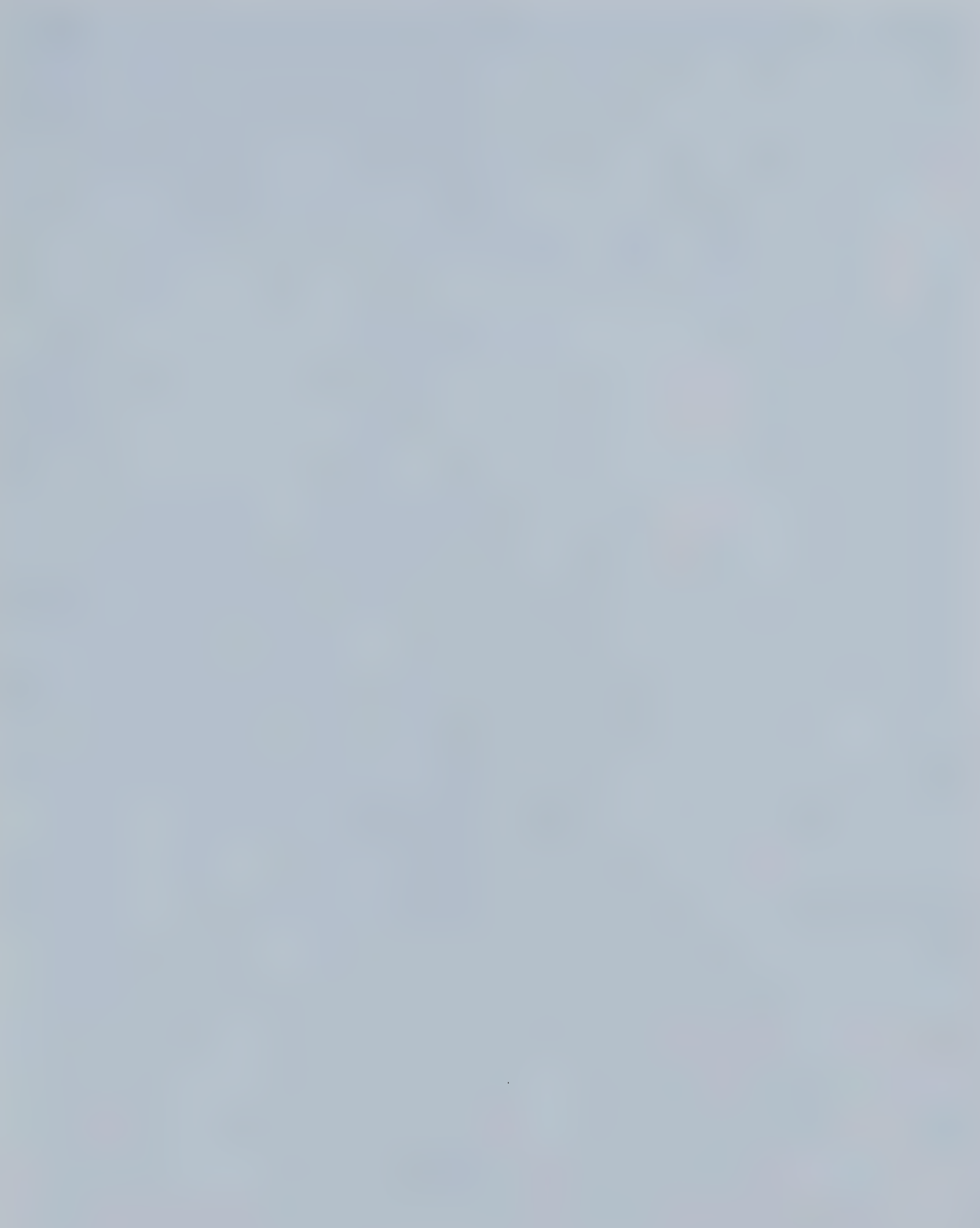


PLATE 36

Figure A. Section of bursa wall, showing formation of macroapocrine secretions (s), cilia (c), apical cluster of mitochondria (m), nucleus (n), and sperm (arrow) in lumen, x8,000.

Figure B. Section through basal portion of several bursa cells, showing pigment (pi) and mucoid (mu) secretion vacuoles, x8,000.

Figure C. Section of bursa wall showing accumulation of apocrine secretions (s) at free borders of cells. Sperm (sp) are visible in lumen, x2,000.

All micrographs made from sections of seminal vesicles of *L. scutulata*.

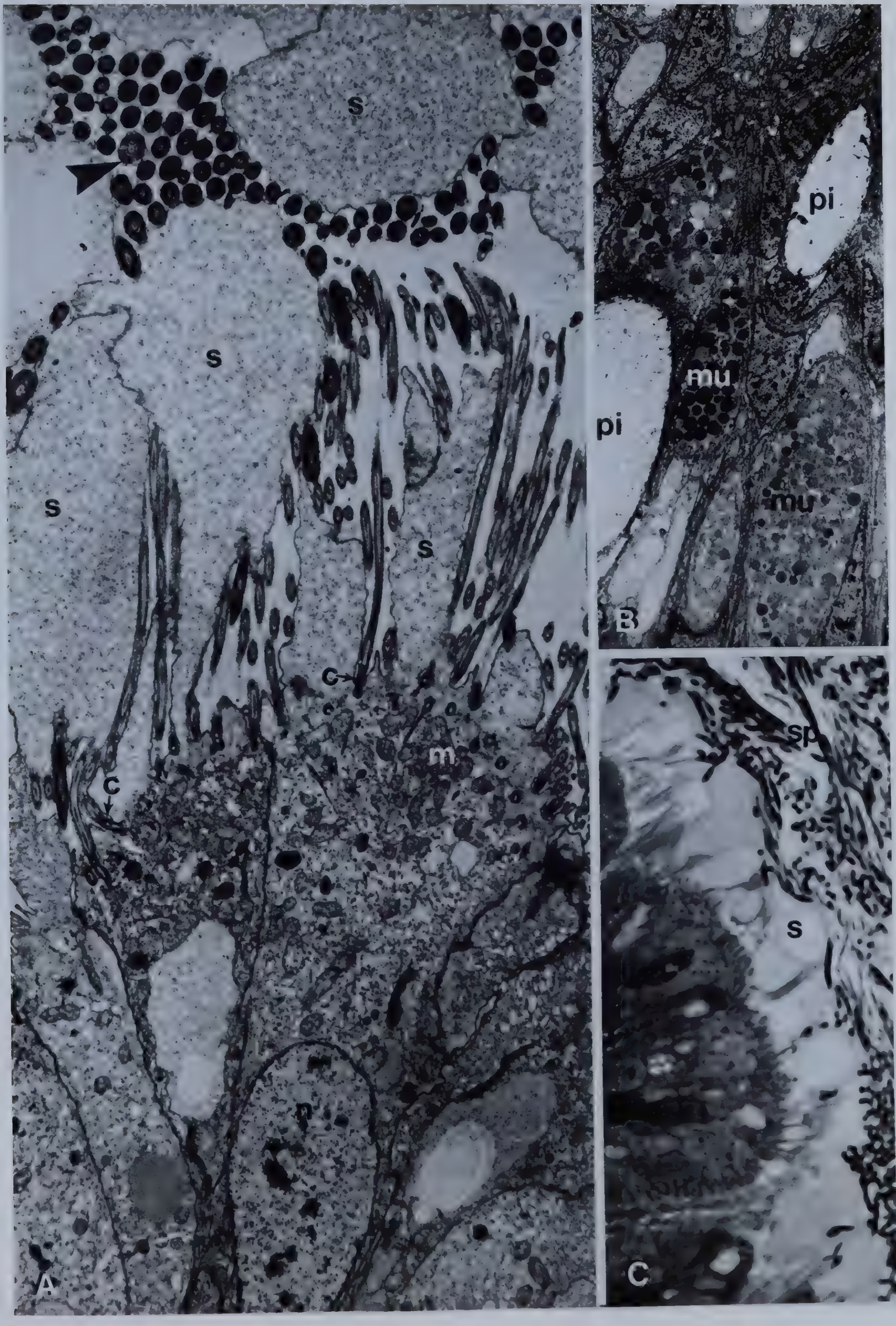

s
s
s
s
c
c
m
n
A
pi
pi
mu
mu
B
sp
s
C

PLATE 37

<u>figure</u> <u>A</u>. Section of bursa wall. Note: formation of macroapocrine secretion (s), microvillar apex with cilia (c), and elongate nucleus (n), <u>x5,000</u>.

<u>Figure</u> <u>B</u>. Light micrograph of section through bursa wall. Note: Pigment secretions (pi), mucoid secretions (arrows), apocrine secretions (s), and sperm (sp) in lumen, <u>x2,000</u>.

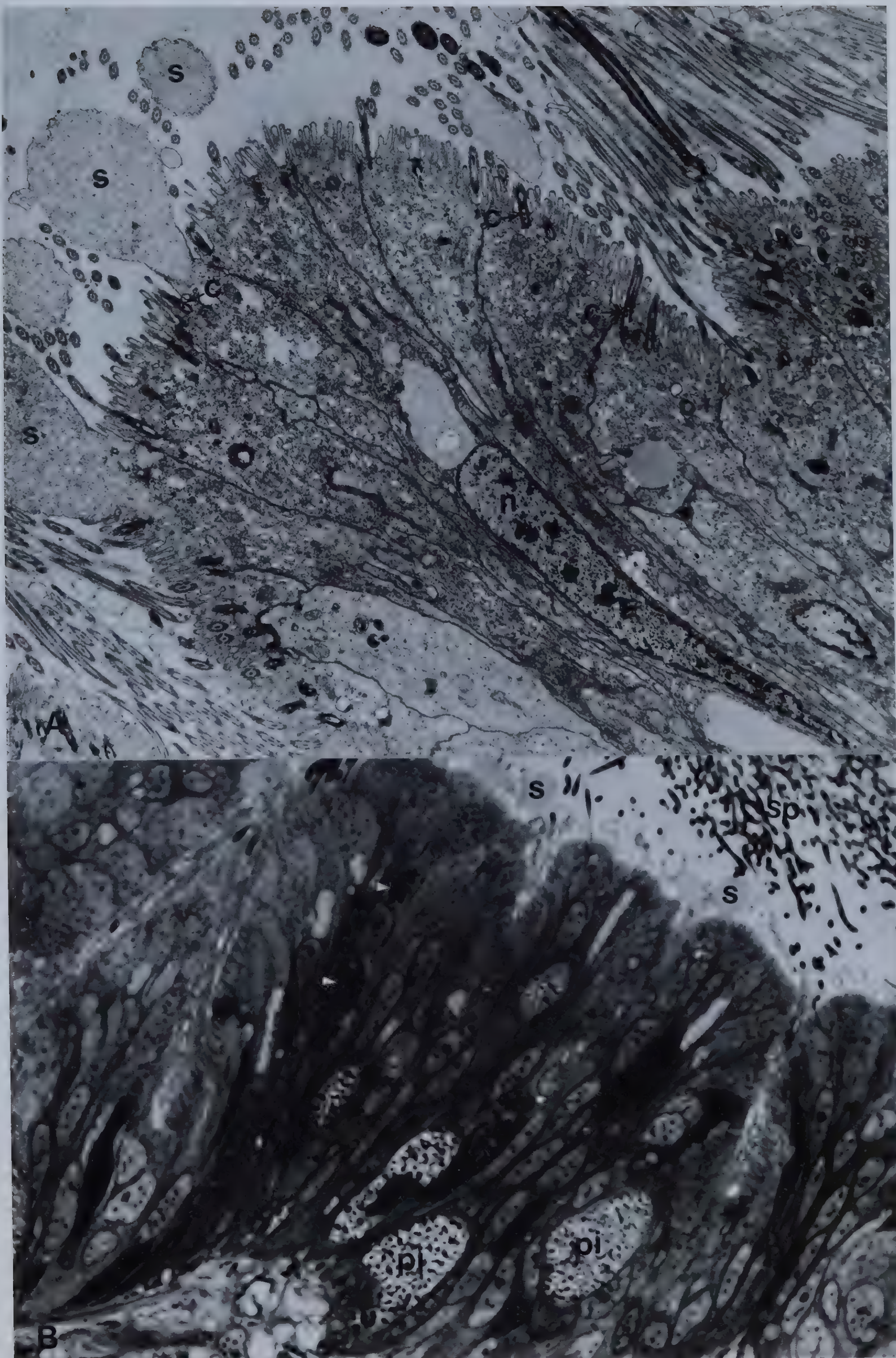

s
s
s
c
c
c
n
A
s
sp
s
pl
pl
B

PLATE 38

Figure A. Sperm heads (arrows) embedded in plasma membrane of receptaculum seminis (seminal receptacle) cell. Mitochondria (m) aggregate at apex of cells, x25,000.

Figure B. Sperm head (arrow) deeply embedded in seminal receptacle cell. Note: aggregation of mitochondria (m) at cell apex, microvilli (mi), golgi bodies (g), x9,000.

Figure C. Low power electron micrograph of seminal receptacle wall, showing general arrangement of cell organelles. Note: median nucleus with nucleolus (n), apical mitochondria (m), residual bodies (r), lipid droplets (li), basal lamina (b), muscle sheath (ms), and sperm (sp), x5,000.

Figure D. Single sperm head (sp) deeply embedded in invagination of plasma membrane (pm) of seminal receptacle cell, adjacent to nucleus (n), x50,000.

All micrographs made from sperm-filled R-S of *L.scutulata*.

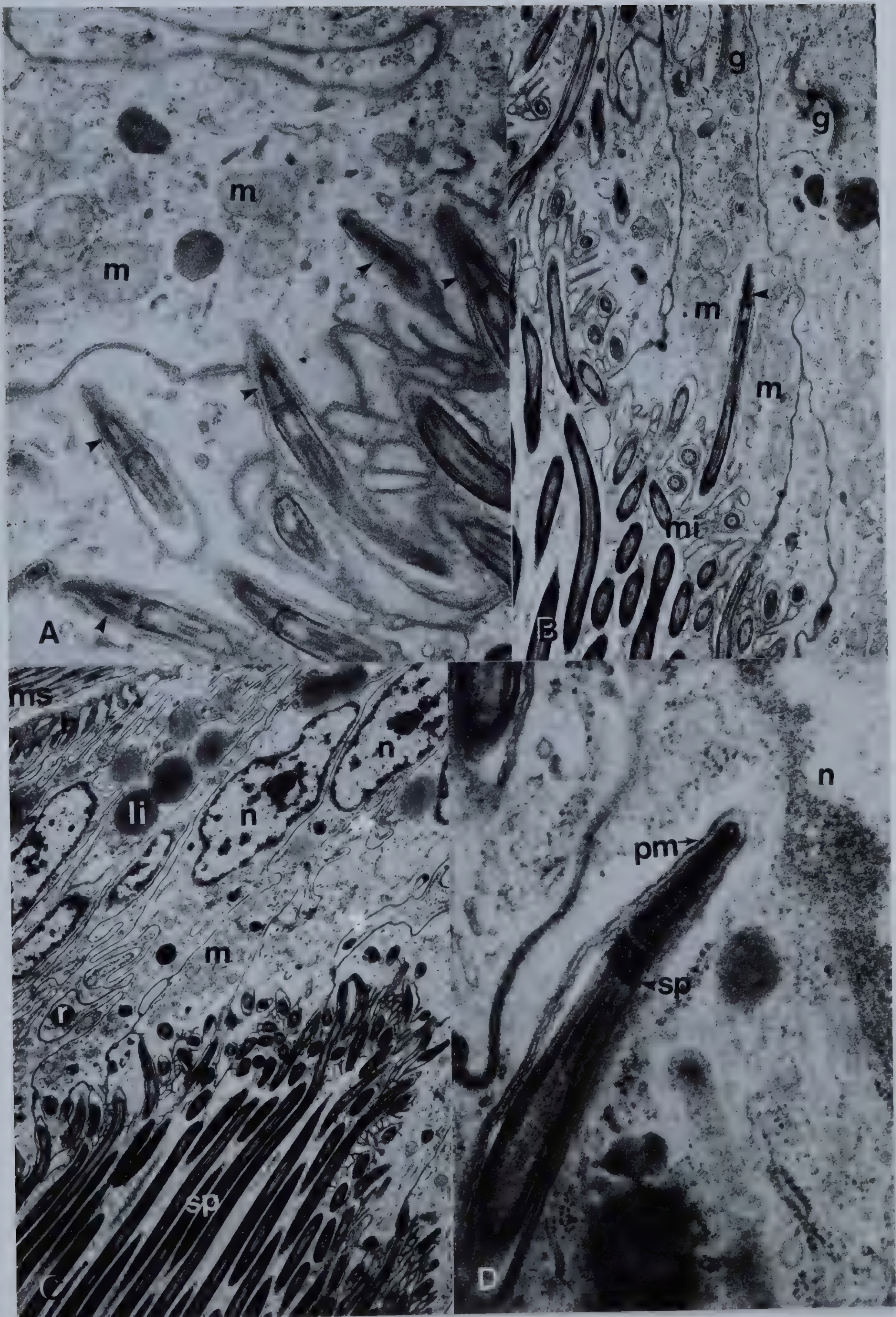

A
m
m
B
g
g
m
m
mi
C
ms
b
li
n
n
m
r
sp
D
n
pm
sp

PLATE 39

Figure A. Low power electron micrograph of albumen gland showing general arrangement of cell contents. Note: Gland cells contain dense secretion granules (s), some of which can be seen forming within whorls of granular endoplasmic reticulum (arrow). Mitochondria, lipid droplets, and large nuclei are also visible. Ciliated cells occupy most of apical surface. Adjacent cells maintain contact via desmosome-like junctions near cell apex, x8,000.

Figure B. L-S through elongate cell processes of jelly gland cells. Secretions are arranged in single file, (total width of micrograph is 190um), x800.

Both micrographs made from sections of pallial oviduct of *L.scutulata*.

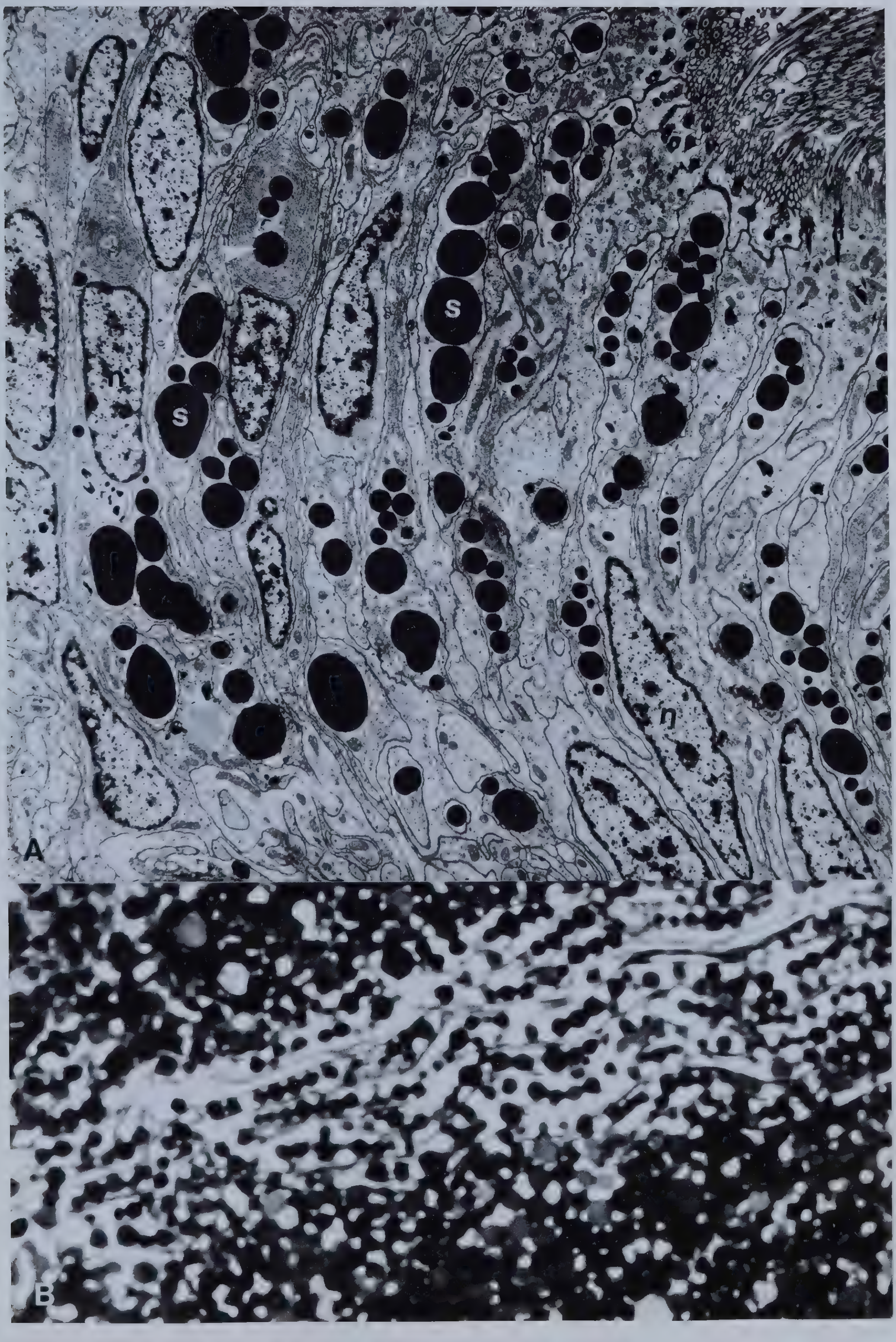

A
B
s
s
n
n

PLATE 40

Figure A. Scanning electron micrograph of the inside of egg capsule wall , showing meshwork of fibres interspersed with droplets of secretion [*L.sitkana*], *x40,000*.

Figure B. Light micrograph of capsule gland cells . At the cell apex, note: gland cell pore (arrow), nuclei of ciliated cells, and dense ciliated surface (c). Numerous secretion granules may be seen in gland cells [*L.scutulata*], *x600*.

Figure C. Light micrograph of jelly gland cells. Note: dense ciliated surface, numerous secretion granules, and pigment secretion (pi) [*L.sitkana*], *x500*.

206

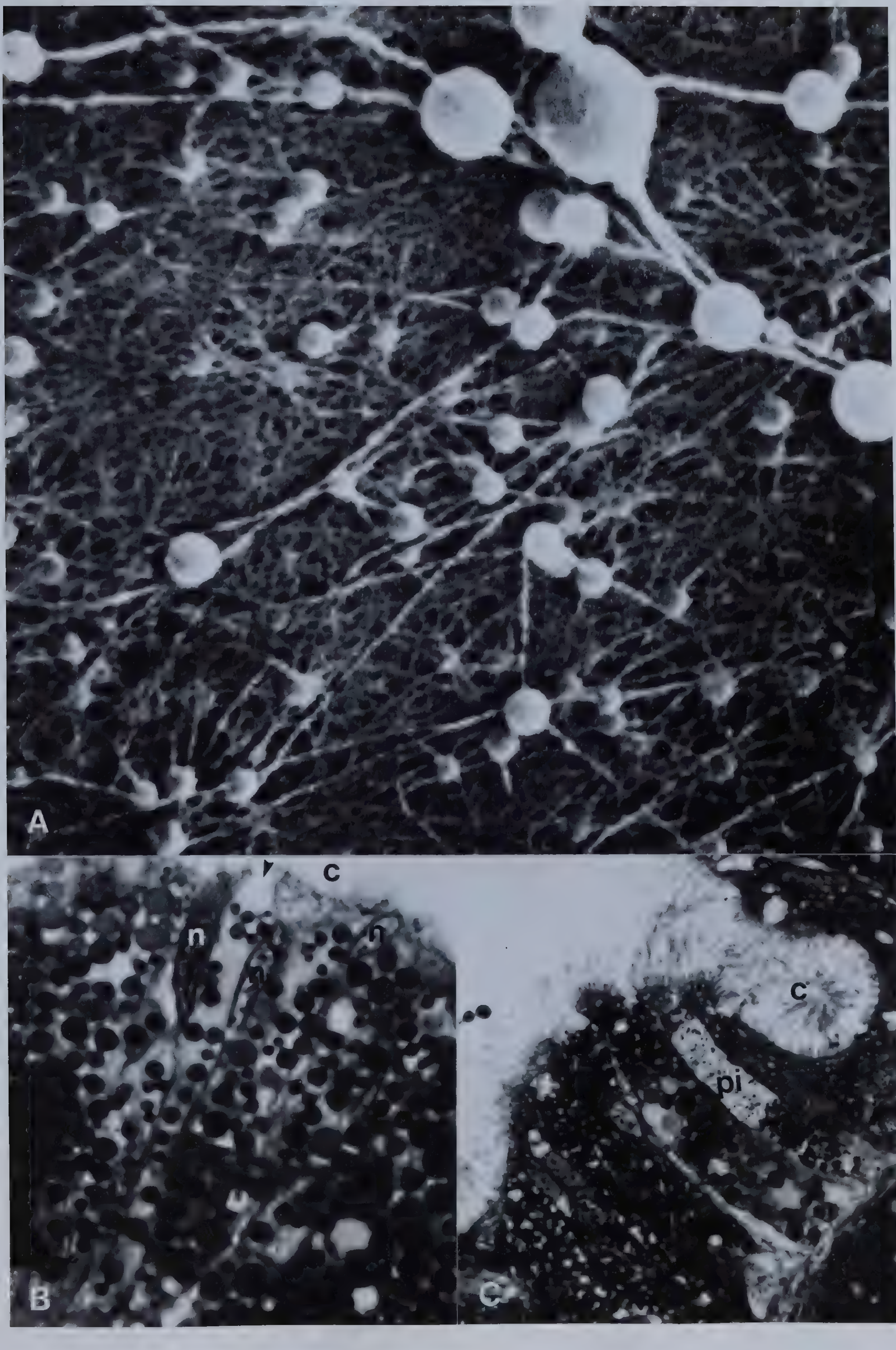


PLATE 41

Figure A. section through covering gland. Note: secretion granules (s), granular endoplasmic reticulum (er), cilia (c). Gland cells empty contents into lumen (lu), via small pores between ciliated cells (x). Adjacent cells maintain contact via desmosome-like junctions (d). In most cells one can see an unusual vesicular body (arrows), x13,000.

Figure B. Section through cells of covering gland, showing the formation of secretion granules (s) within whorls of granular endoplasmic reticulum, x13,000.

Figure C. High power electron micrograph of vesicular body, showing long vesicle (large arrow), and microtubules (small arrow), . The membranes of the vesicular body measure only 55A compared to 75A of the adjacent plasma membrane (pm), x93,000.

All micrographs made from sections of ripe Covering Gland of *L.scutulata*.

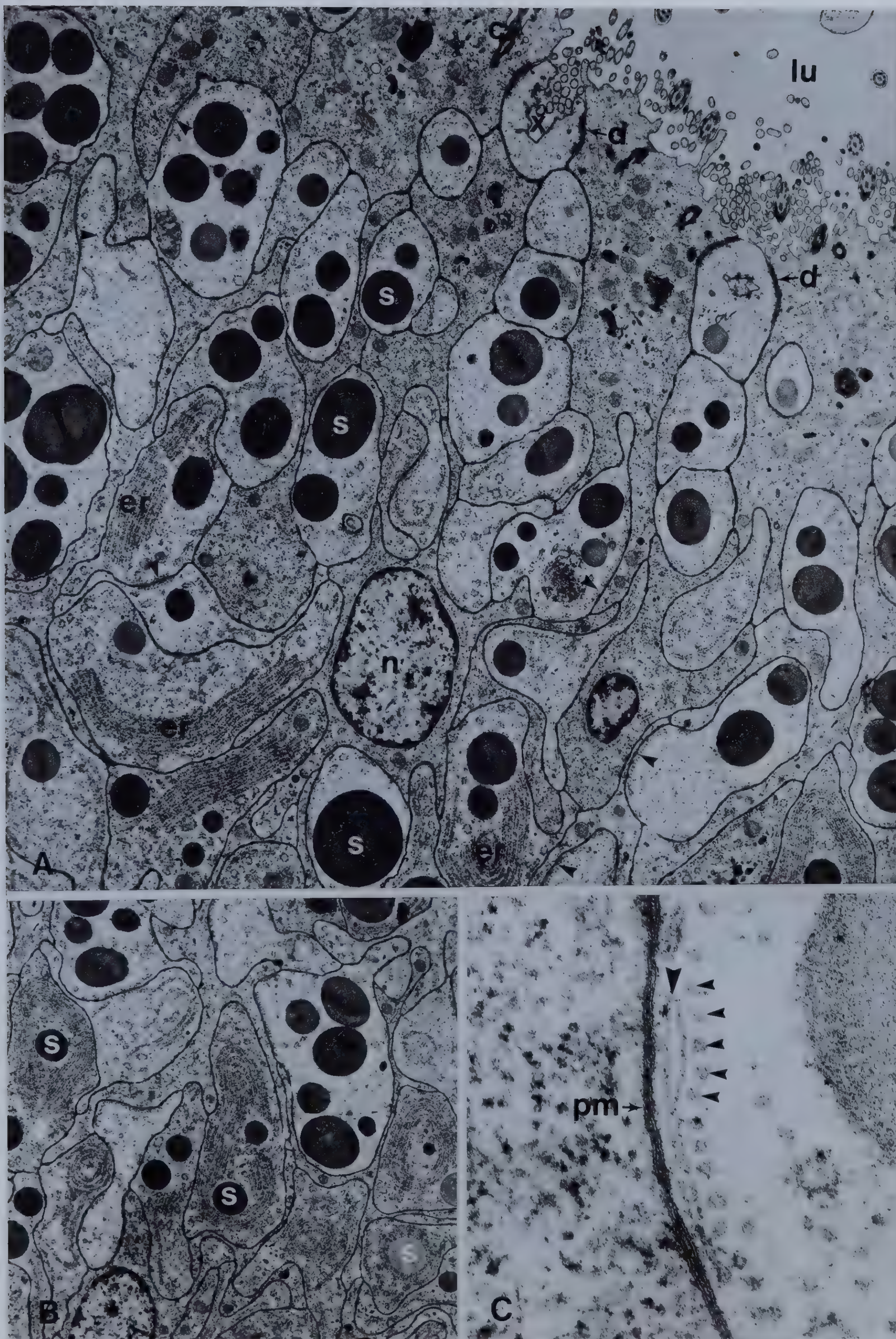

lu
d
d
s
s
er
er
n
s
er
A
s
s
s
B
pm
C

PLATE 42

Figure A. Light micrograph of section through capsule gland of *L. sitkana*, stained by Masson's trichrome. Note: mucoid secreting region (M, stained blue), and protein secreting region (p, stained red), x100.

Figure B. Light micrograph of section through egg capsule of *L. sitkana*, stained by Masson's trichrome. Note: mucoid component (m, stained blue); and proteinaceous component (p, stained red), x115.

Figure C. Light micrograph of section through egg capsule (ec) in jelly gland of *L. sitkana*, stained by AB-AY. Note: fibrous jelly secretion (J, stained greenish-blue) around egg capsule, x350.

Figure D. Light micrograph of section through egg capsule of *L. scutulata* stained by Masson's trichrome. Note: yolk (y) is surrounded by an egg covering (arrow). Albumen, which stains blue with this technique, is not visible between the two layers, x340.

Figure E. Light micrograph of section through an egg capsule of *L. scutulata* in the process of formation in the capsule gland. Notice how the walls of the gland form the basic saucer shape of the egg capsule (arrows). The final shaping is done in the vestibule, x200.

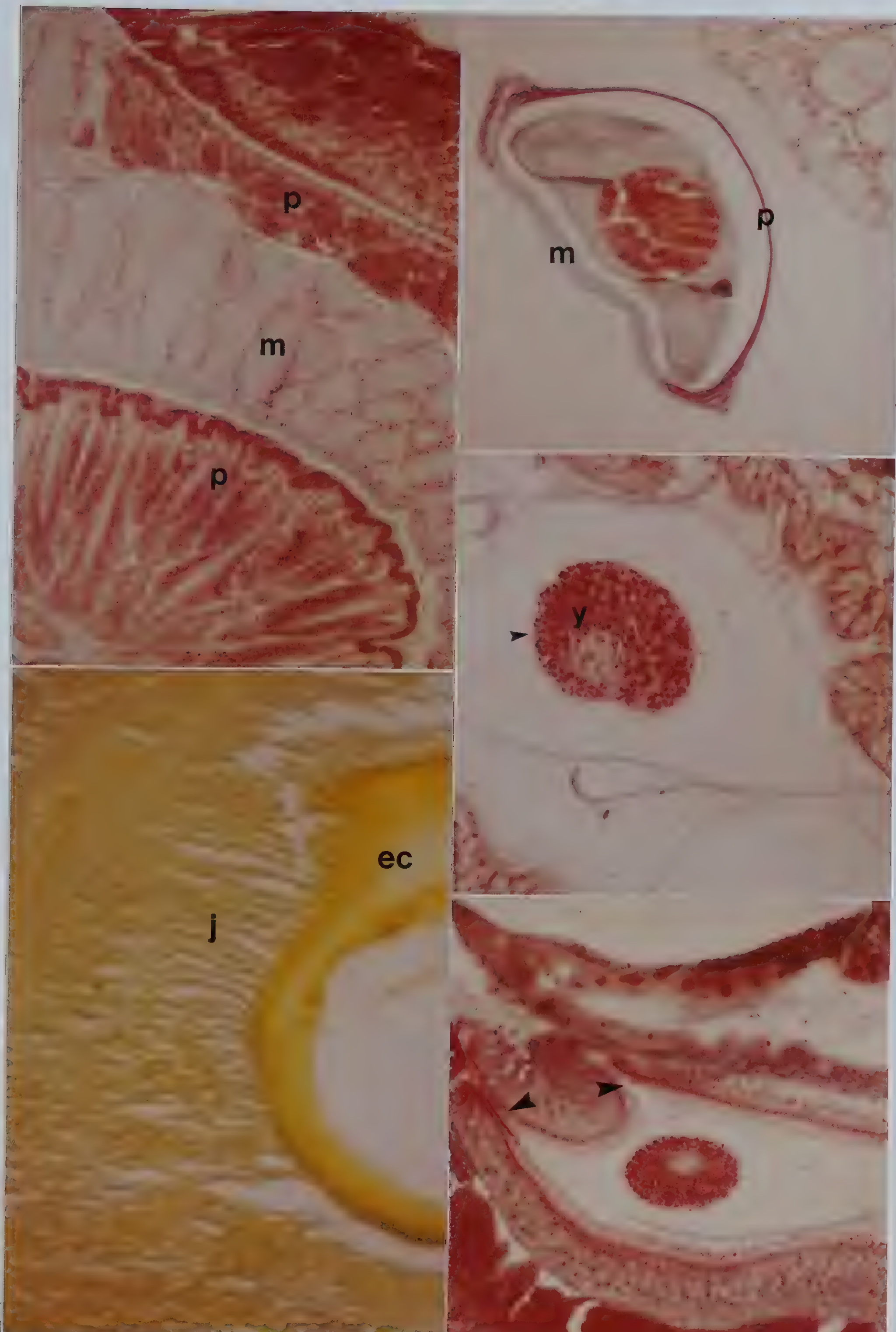

p
m
p
p
m
y
ec
j

PLATE 43

Egg capsule of *L.scutulata* showing egg coverings of four embryos interconnected by fibrous strands, *x24*.

PLATE 44

Seasonal fluctuation in egg production of caged *L.sitkana* over a period of 3 years (1969 - 1973), versus the rate of day length changes during the year. Thanks to Sylvia Behrens for supplying the 1969 data.

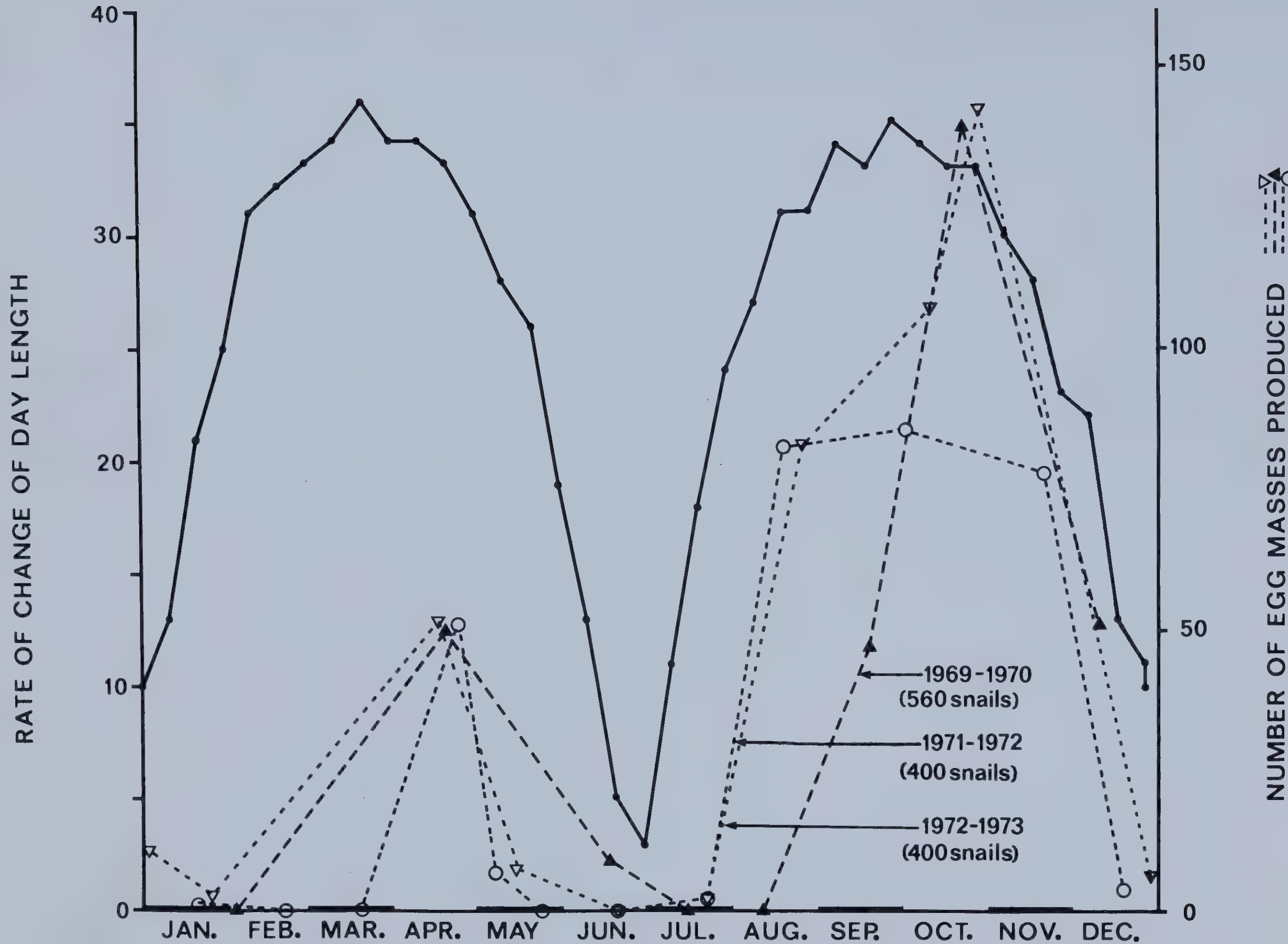

RATE OF CHANGE OF DAY LENGTH
NUMBER OF EGG MASSES PRODUCED
0
10
20
30
40
50
100
150
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
AUG.
SEP.
OCT.
NOV.
DEC.
1969-1970
(560 snails)
1971-1972
(400 snails)
1972-1973
(400 snails)

PLATE 45

Early Larval Development of L.sitkana

Figure A. Formation of first polar lobe (pl) and fertilisation membrane (fm), x150.

Figure B. Formation of polar bodies (pb) and polar lobe (pl) x45.

Figure C. Zygote , 1-cell stage , x50.

Figure D. 2-cell stage, x50.

Figure E. 4-cell stage with polar lobe (pl) attached to D cell (lower left), x50.

Figure F. 8-cell stage showing spiral arrangement of 4 micromeres upon 4 macromeres, x50.

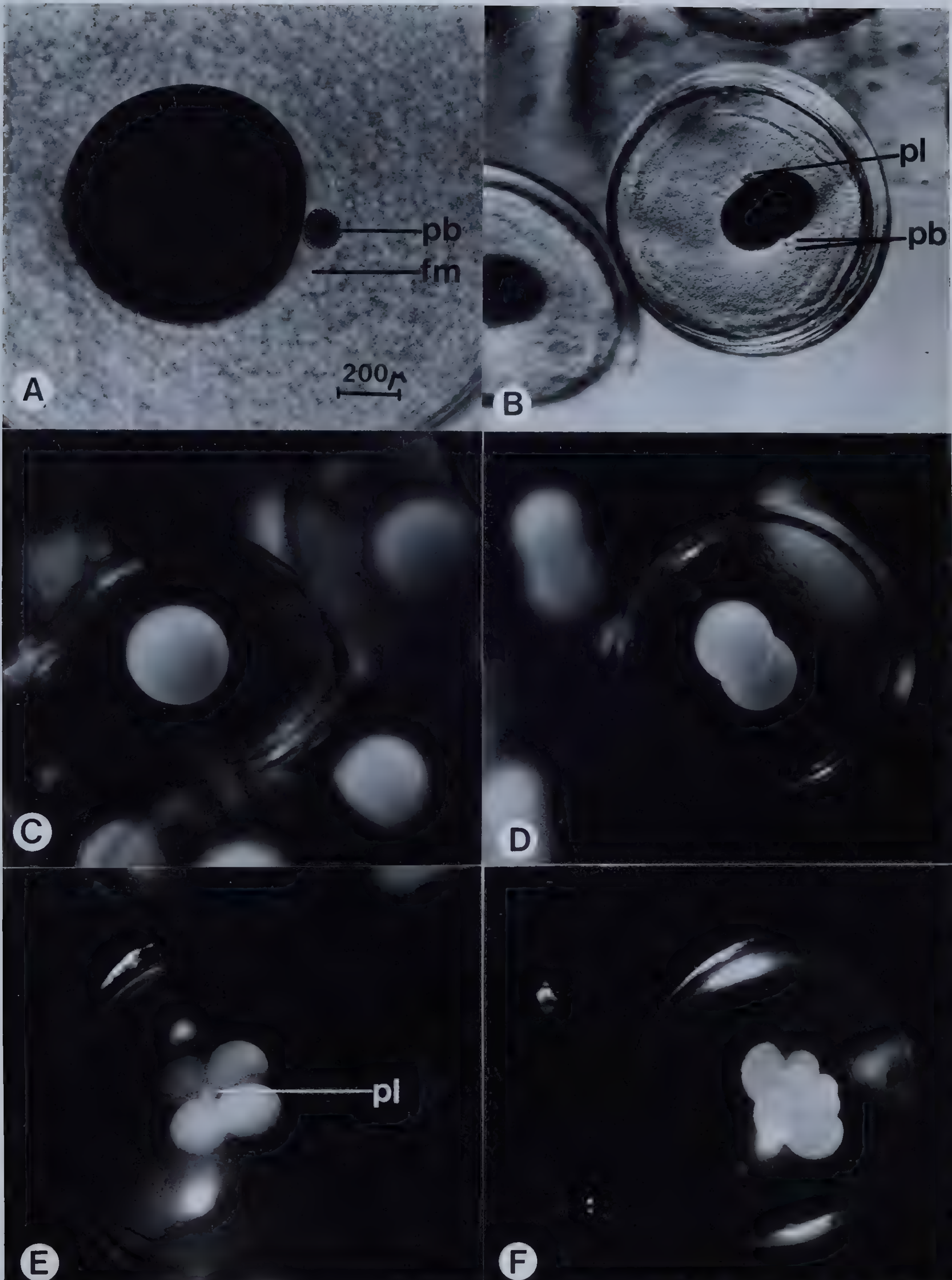

pb
fm
200μ
A
pl
pb
B
C
D
pl
E
F

PLATE 46

Late Larval Development *L.sitkana*

Figure A. Late gastrula stage showing site of stomodaeum (st), *x50*.
Figure B. Very young veliger still contained by fertilisation membrane within albumen, *x65*.
Figure C. Young veliger emerging from albumen (al), *x55*.
Figure D. Young veliger outside albumen, *x55*.
Figure E. Veliger with cup-shaped larval shell (arrow), *x50*.
Figure F. Late veliger showing orientation within capsule, *x55*.

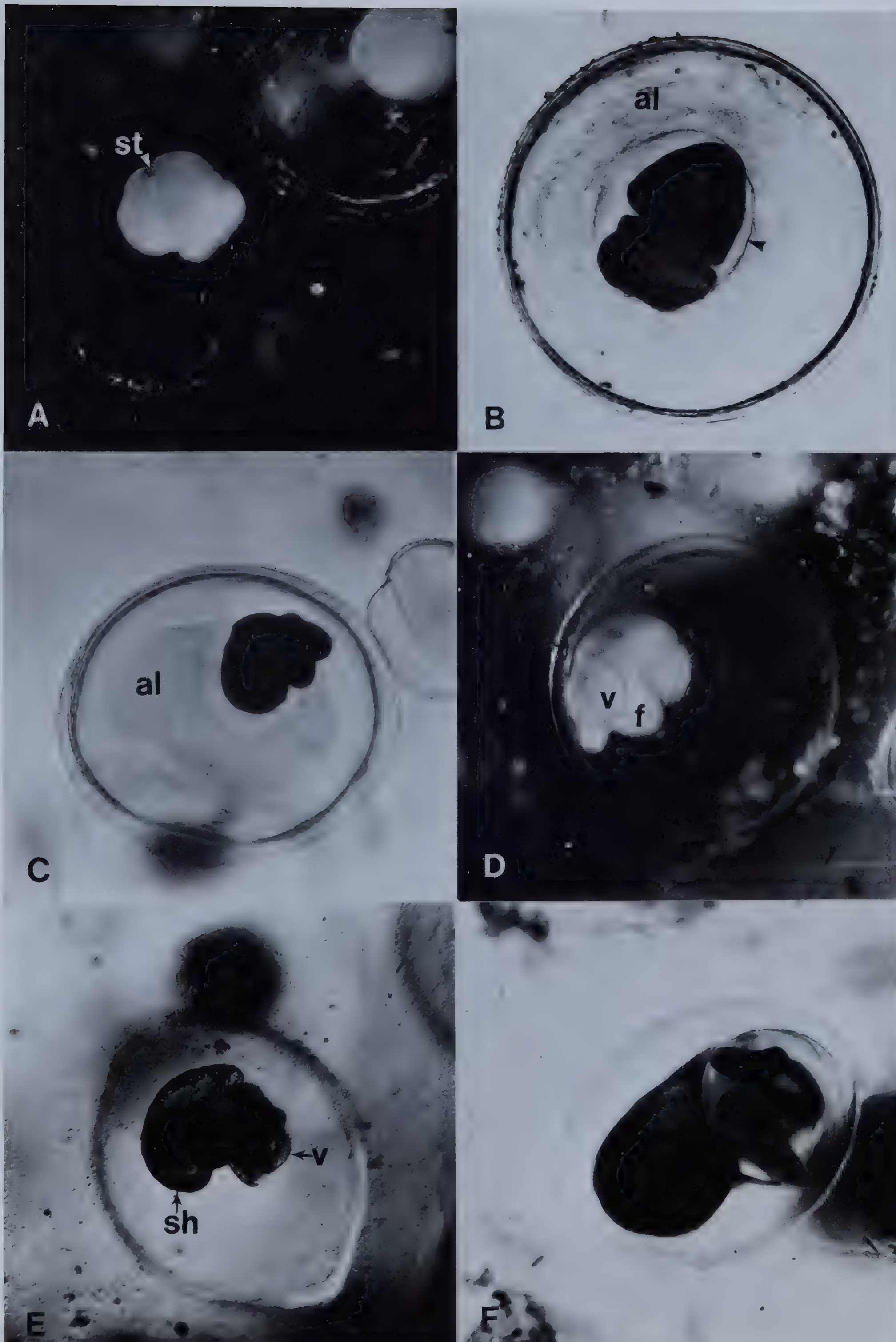

st
A
al
B
al
C
v
f
D
v
sh
E
F

PLATE 47

Hatching in *L.sitkana* I

Figure A. Pre-hatching juvenile. Note: egg covering (arrow) is being drawn away from inside of capsule, *x65*.

Figure B. The juvenile has ruptured a hole in the capsule wall, *x60*.

Figure C. The juvenile has succeeded in getting one tentacle and most of the foot through the ruptured capsule wall, *x60*.

Figure D. The head and tentacles have been pulled through the break in the capsule wall, *x60*.

Figure E. The foot gains a hold on the outside of the capsule and begins pulling the rest of the body out after it, *x60*.

Figure F. The shell and visceral mass are being pulled through the hole in the capsule wall by the foot. Hatching was completed a few seconds later, *x60*.

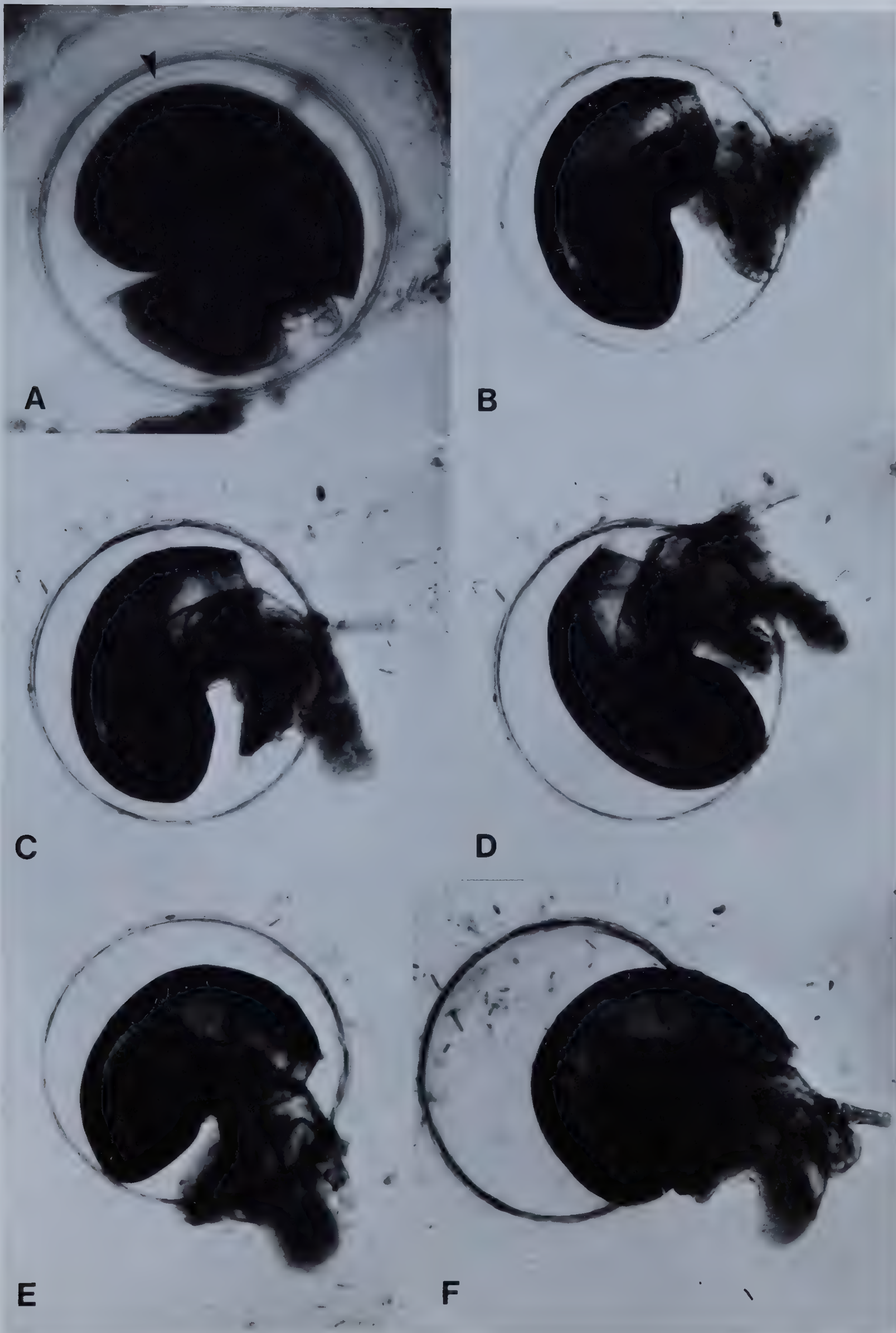

A
B
C
D
E
F

PLATE 48

Hatching in L.sitkana II.

Figures A - F. Similar sequence of events as shown in PLATE 47, except that hatching has been prolonged and the capsule has become much more flexible (note, Figures B - D). After emergence of the snail, much of the capsule wall dissolves indicating the presence of a substance that mediates hatching, x60.

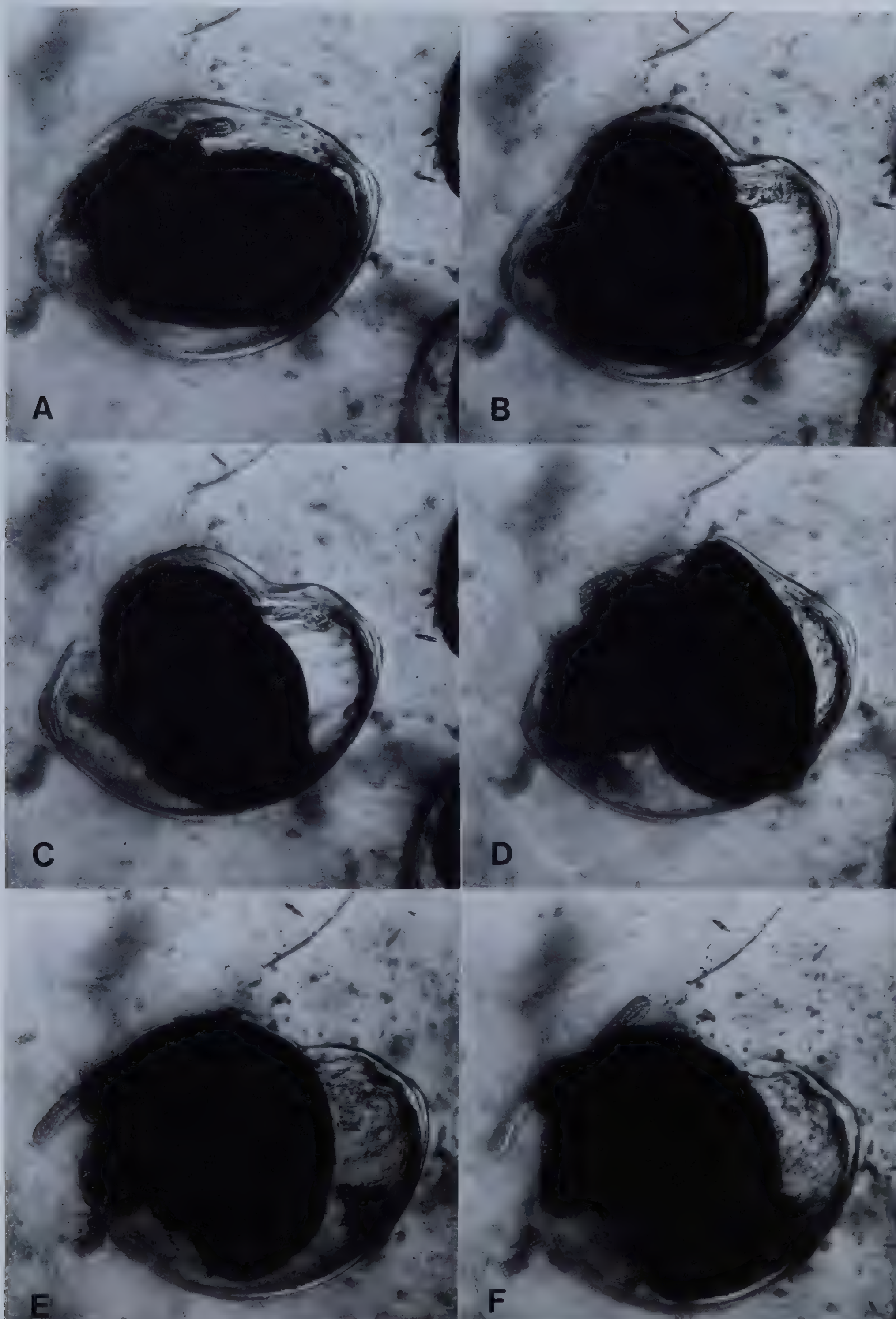
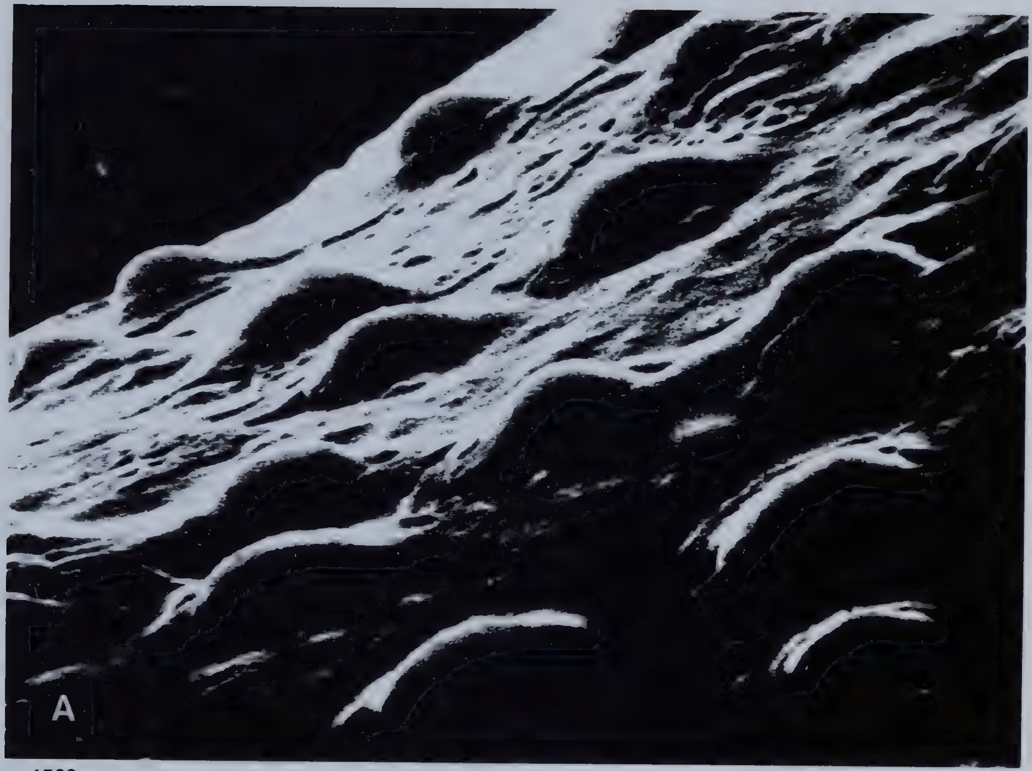

A
B
C
D
E
F

PLATE 49

Figure A. Scanning electron micrograph showing the tubercles on the larval shell of *L. sitkana*, x10,000.

Figure B. Growth of embryos and juveniles of *L. sitkana*. Each circle represents the mean of 15 measurements. The size indicates either the diameter of the embryo, or the shell length of veliger (or juvenile). Point V (arrow) indicates the time when the veligers began to feed on the albumen and point H (arrow) indicates the approximate time of hatching of the juveniles.


A

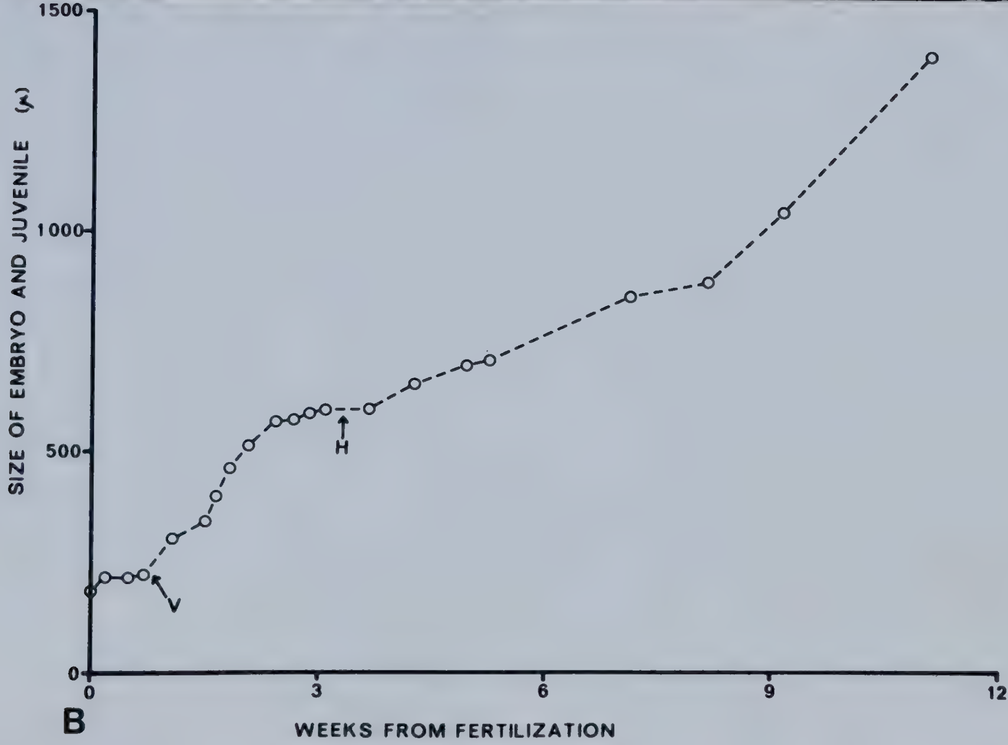
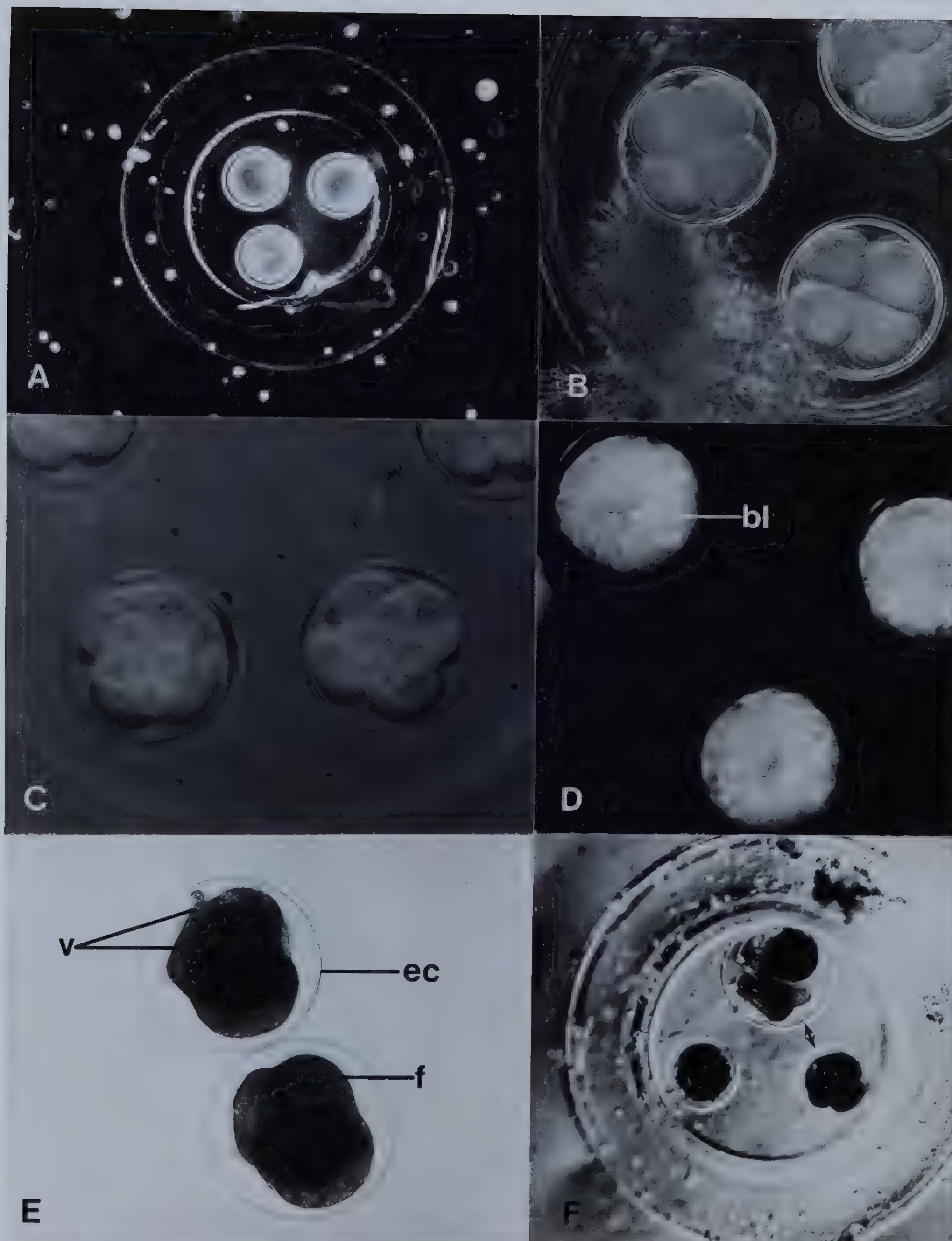

1500
1000
500
0
SIZE OF EMBRYO AND JUVENILE (μ)
V
H
0
3
6
9
12
B
WEEKS FROM FERTILIZATION

PLATE 50

Larval Development of L. scutulata

Figure A. Egg capsule containing three eggs viewed from above, x70.
Figure B. 4-cell stage, x170.
Figure C. 8-cell stage, x180.
Figure D. Gastrula stage showing the irregular outline of the blastopore (bl), x170.
Figure E. Young veligers showing the foot (f) and velum (v). Note: expansion of the egg membrane (em), x165.
Figure F. Pre-hatching veliger and two immature veligers showing how the egg membrane has expanded in the pre-hatching stage (double arrow), x90.


A
B
C
D
E
F
bl
v
ec
f

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APPENDIX 1

APPENDIX 1

Hatching Success of Larvae Subjected to Different Environmental Factors

Preliminary experiments were set up to test the effects on hatching success of temperature, the sulphide system and diatom growth on egg masses.

Egg masses were cultured to the late gastrula stage on a diatom-infested substrate and on a diatom-free substrate. For each experiment equal numbers of egg masses of similar size were separated into control and experimental vials. New, 50 ml, plastic-capped vials, which had been rinsed with filtered sea water, were used throughout the experiments.

1. Diatom Experiment

The purpose of this experiment was to test my hypothesis that a symbiotic relationship exists between veligers and diatoms due to local concentration gradients of carbon dioxide (produced by the veliger's metabolism benefitting the diatoms) and of oxygen (produced by diatom photosynthesis benefitting the veligers). A literature search showed that in the past several workers had found that algae growing in *Ambystoma maculatum* egg masses formed a symbiotic relationship with the salamander embryos (Gilbert, 1942, 1944; Hutchison and Hammen 1958). A review of other symbiotic relationships of this nature in

invertebrates was published by C.M. Yonge (1944).

The partial pressure of oxygen in sea water (pOx) was measured with a Radiometer Blood Analyser at the beginning of each experiment to ensure a low pOx in experimental vials. Experiments were conducted in the light and unfortunately there was no time to duplicate others under dark conditions.

One set of vials (control/experimental) contained egg masses without diatoms and the second set with diatoms. Controls contained filtered sea water at pOx 160 mm Hg and experimentals were at pOx 40 mm Hg.

Results - See Table 4A

Discussion and Conclusions

The experiment indicates that the diatom layer did not enhance the survival of embryos at very reduced oxygen tensions. However intermediate conditions should have been set up to see if there was a gradation of effect. Also an 'in dark' experiment should have been conducted. The oxygen analyser was not available at the end of the experiment to measure final pOx.

The experiment also indicates the marked dependence of embryos on oxygen availability. All egg masses developed to the veliger stage indicating that this time of increased activity has higher oxygen requirements, which is to be

expected.

The fauna of the egg envelope becomes extremely varied after a few days in the field. Besides an abundant growth of diatoms and unicellular algae one can also find nematodes; nechtochaetal larvae and other annelids; various crustaceans; numerous protozoa (some of which gain access to the interior of the egg capsules via pores in the fibrous egg envelopes); bacteria; and juvenile snails. Without limiting these variables, all of which may affect local concentrations of carbon dioxide and oxygen, one cannot demonstrate an interrelationship between photosynthetic organisms in the egg envelope and veligers in the egg capsules.

2. Temperature Experiment (*L.sitkana*)

The purpose of this experiment was to test the effect of temperature on hatching success within environmental limits. It has been shown that egg masses of *L.sitkana* exposed above the water level in tide pools are subject to desiccation and subsequent death of embryos, (Behrens 1972). However it was not known if the embryos died from either water loss, or increased salinity or temperature, or all of these factors.

Egg masses without diatoms were used. One vial was set up as a control (together with one from the previous experiment) and was maintained on the water-table at 10°C.

Five other vials were subjected to a gradation of temperatures (20°, 25°, 30°, 32°, 34°C) on a thermostatically controlled water-bath. Vials were capped to prevent excessive evaporation and the experiment was run for a fixed period (6 hours) before changing the water and returning to the watertable.

Results - See Table 4B

Discussion and Conclusions

The results show that the embryos are independent of temperature within the limits tested (maximum water temperature observed at False Bay 30°C) provided salinity is kept constant, (c.f. Southward 1958).

Since salinity, water loss and temperature are interdependent it is suggested that their combined effects cause the death of exposed embryos. this data is supported by Behren's (1970). The idea had been previously proposed for other gastropods in the terms of a Dessication/Heat/Light complex (Broekhuysen, 1940; Evans, 1947; Stephenson, 1944; Southward, 1958).

Sulphide System Experiment

The purpose of this experiment was to test the effects of a 'black sand' substrate on the hatching success of embryos. Black sand is indicative of decaying animal and vegetable matter, and a virtually anaerobic environment

usually associated with the release of hydrogen sulphide gas (Fenckel and Reed, 1970).

Egg masses without diatoms were used. Three egg masses were introduced into each of three vials. One vial had no sandy substrate, a second had clean sand and the third had black sand. All vials were kept on the water table at 10°C and the water was changed daily.

Results - See Table 4C

Discussion and Conclusions

Black sand is lethal to the embryos but it is not known whether this is due to the toxic effect of dissolved hydrogen sulphide gas and chemical products of degeneration, or whether it is due solely to the anaerobic environment present in such situations. The result with 'clean' sand was unexpected and obviously this substrate was also highly unfavourable. However I suspect that the 'clean' sand was in fact not clean and created a more anaerobic environment as the experiment proceeded. This substrate should have been prepared by autoclaving some 'clean' sand to kill all the bacteria present, thereby testing the effect of the substrate itself.